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CORRECTIONS

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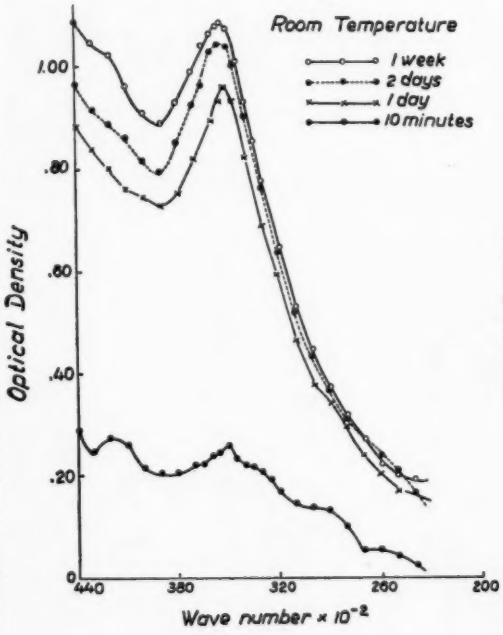
Page 959. In the Abstract, line 14, the sentence "The rate of repolarization was decreased . . ." should read "The rate of repolarization was increased . . .".

Page 1181. The first column of Table I should appear as follows:

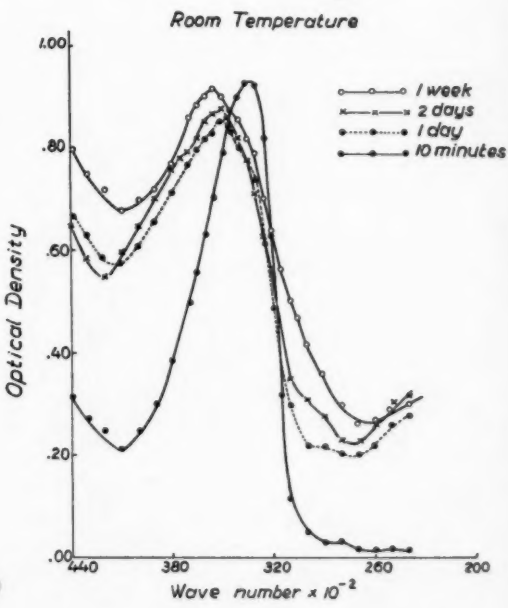
(1) Albumin*
Conjugate
(2) Albumin
Conjugate
(3) Albumin
Conjugate
(4) Albumin
Conjugate
(5) Albumin
Conjugate

Pages 1227, 1228. The lower figures on these two pages should be transposed; the figure that appears on p. 1227 is 4*b* and the figure on p. 1228 is 3*b*, as indicated below.

3*b*



4*b*



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VOLUME 37 MARCH 1959 NUMBER 3

THE SITES OF TRANSFER OF SODIUM, POTASSIUM, AND IODIDE IN THE PAROTID DUCT SYSTEM OF THE DOG[1]

A. S. V. BURGEN, K. GODWIN TERROUX, AND E. GONDER

Abstract

Rapid intra-arterial injections of radioactive iodide, sodium, and potassium have been made into the carotid artery while the parotid gland was secreting saliva at a steady rate. All three ions appeared in the saliva within a few seconds with iodide lagging slightly behind sodium and potassium. The curve for potassium was several times as long as those for sodium and iodide. With intravenous injections of the isotopes, after sufficient time had been allowed for equilibration between the blood and the parotid gland, there was an initial transient concentration of all three ions when secretion started after a rest period. The transient concentrations for sodium and potassium appeared slightly ahead of that for iodide. Potassium showed a clear second phase in its rest transient concentration.

It is concluded that all three ions are able to cross the salivary duct epithelium and enter the saliva. Evidence for active transfer of potassium into the salivary duct lumen is presented.

Introduction

In a previous paper (1) an analysis of the specific activity relationships of radioactive sodium, potassium, and urea in the salivary gland and in its secretion led to the conclusion that salivary secretion was a composite process. The theory put forward to account for the experimental results obtained was that a primary secretion, which was greatly modified as it passed down the salivary ducts, was produced in the acini. In particular, it appeared that much of the potassium in the primary secretion was lost by passage across the duct walls and that nearly all the sodium in the secreted saliva was sodium that had entered the saliva as it passed down the ducts. In other experiments (2) it was found that when an animal had been given radioactive iodide and the parotid gland had been subsequently brought into activity by nerve stimulation, the earliest samples of saliva had a considerably higher concentration of iodide than the later samples. Various arguments were adduced to support the conclusion that this concentration transient was due to iodide accumulating in the duct lumen during the rest period.

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Contribution from the Department of Physiology, McGill University, Montreal, Que. Aided by a grant from the National Research Council of Canada.

It is indeed obvious that if the duct system does not collapse completely during inactivity, the first secreted saliva must always be the resting duct contents expelled in advance of the newly formed saliva.

This paper describes experiments utilizing more delicate methods of saliva analysis in order to distinguish the sites at which the three ions sodium, potassium, and iodide cross the duct epithelium, and also analyzes in more detail the transient concentration levels of these ions following the onset of secretion.

Methods

The methods of dissection and preparation of the gland have been described previously (3). Rapid changes in the composition of the saliva were followed by allowing each drop of saliva to fall on a slowly moving strip of filter paper; a time signal marked 2-second intervals. The filter paper was later dried in an oven at 110° C for 20 minutes and after the drops were numbered and the time at which they were formed was recorded, the strip was either cut into strips for scintillation counting or punched into 1-in. diameter disks for Geiger counting. The paper used was Whatman 3MM chromatography paper and in order to render the saliva spots readily visible after drying it was sprayed with a solution of 0.01% bromphenol blue in 20 m*M* sodium bicarbonate and dried before being used for collecting drops. The drops of saliva appeared as well-defined blue-ringed circles on the paper.

It was found that if the exterior of the salivary cannula tip was smeared lightly with silicone grease the drop size remained remarkably constant (variation less than 5%). The volume of each drop was measured by weighing 20 drops collected in a tared bottle. The accuracy of counting with drops applied to paper in this way was nearly as good as planchet counting in the case of β-counting and was equal to that with dissolved samples in the case of scintillation well counting.

Standards were prepared by micropipetting suitable volumes of the stock isotope solutions directly onto filter paper prepared as for drop collection. In most of the experiments to be described two isotopes were given together so that a comparison of their behavior might be made. Actually I^{131} ($t\ \frac{1}{2}$ = 8.04 days) was used in all experiments in combination with either K^{42} ($t\ \frac{1}{2}$ = 12.6 hours), Na^{22} ($t\ \frac{1}{2}$ = 2.6 years), or Na^{24} ($t\ \frac{1}{2}$ = 15.1 hours).

The methods used were as follows:

I^{131} and K^{42}.—GM counting with a thin end-window tube (Tracerlab TGC2)

(*a*) Immediate counting, followed by counting after 4 to 8 days at which time K^{42} activity had decayed to very low levels. The amounts of K^{42} and I^{131} were calculated by solution of the simultaneous equations so obtained. This method was tedious, and accurate only when the amount of the long-life isotope (I^{131}) was relatively small in comparison with the short-life isotope (K^{42}).

A more satisfactory alternative method was:

(*b*) Initial counting with a β-filter adequate to reduce the lower energy (0.606, 0.335 Mev) radiation from I^{131} to less than 5%. This filter reduced the counting rate from the much higher energy radiation (3.55, 1.99 Mev) of K^{42} by about 20%. The immediate count was thus almost exclusively from K^{42}. About 1 week later the samples were recounted without the filter. At this time K^{42} had decayed to less than 0.1% of its initial activity, whereas the I^{131} had decayed by 50%; the counts were therefore primarily due to I^{131}. The mathematics in this method were much less cumbersome and accurate results could be obtained over a wide range of isotope ratios.

I^{131} and Na^{24}

The second method used for K^{42} and I^{131} was equally useful with I^{131} and Na^{24}. Additional efficiency could be obtained by scintillation counting if the increased background was acceptable.

I^{131} and Na^{22}

The similarities in β-ray energies of these two isotopes made the second method inapplicable and the most satisfactory results were obtained with a scintillation well counter, recounting after decay of the I^{131} by 2 to 4 half lives (i.e. 2–4 weeks). In these experiments it was desirable to have a higher counting rate due to I^{131} than to Na^{22}.

Na^{24}, K^{42}, and I^{131} were supplied by Atomic Energy of Canada Ltd., through Charles E. Frosst and Co., Montreal. Na^{22} was obtained from AERE, Harwell, England.

The isotopes were administered as follows: For intra-arterial injection the central end of the thyroid artery was cannulated with a blunt metal cannula whose tip was advanced until it lay just within the lumen of the common carotid artery. Rapid injections of isotopes were made after the gland had been secreting at a steady rate for a minimum of 1½ to 2 minutes. At this time the ionic composition of the saliva had become reasonably constant. The total volume injected was 0.5 ml, containing

(1) 10 μc I^{131} and 10–20 μc Na^{22}
or (2) 1–2 μc I^{131} and 1–20 μc K^{42}.

For intravenous injections containing I^{131} and K^{42}, the isotopes were injected on a schedule of decreasing infusion rates which, on the basis of previous experience, had been found to result in the maintenance of substantially constant plasma levels during the stimulation periods. The isotopes were given in two steps: (*a*) a loading dose of 4 to 5 mc K^{42} and 50 μc I^{131} injected during a period of 15 to 20 minutes; (*b*) a sustaining infusion containing 4 to 5 mc K^{42} and 5 μc I^{131} injected slowly for a further period of up to 2 hours. Stimulation was started only after a minimum of 1 hour from the start of the infusion. Before collection of saliva for analysis was started, the isotope in the gland was equilibrated with that in the blood by two periods of stimulation, each for 4 minutes. When I^{131} and Na^{24} were given together, a single injection containg 50 μc I^{131} and 5–10 mc Na^{24} was used and a period of 20 to 30 minutes allowed for equilibration before stimulating the gland.

Loss of K^{24} in the urine was prevented by tying both ureters. Formation of protein-bound iodine was prevented by the administration of 50 mg/kg mercaptazole (Tapazole, kindly supplied by Eli Lilly and Co.) previous to the injection of iodine.

Results

Part I. Intra-arterial Injection of Radioactive Ions while the Gland Was Secreting Saliva at a Steady Rate

(a) Sodium (Na^{22} or Na^{24}) and Iodide (I^{131})

A typical example of the kind of curve obtained from the parotid gland is shown in Fig. 1. An intra-arterial injection of 16 μc Na^{22} and 10 μc I^{131} in a volume of 0.5 ml was made at time zero and was completed in less than 1 second. Saliva was being secreted at a rate of 0.31 ml/g minute.

An abrupt appearance of both isotopes in the saliva occurred after a few seconds, sodium appearing at 5.5 seconds slightly ahead of iodide at 7.0 seconds. The concentration of both isotopes rose quickly reaching 90% of the peak height in 6 seconds for Na and 6.5 seconds for I. After a brief peak of a few seconds the concentration declined quite rapidly. The duration measured by the width of the curve at half maximum height was 20.5 seconds for Na and 24.5 seconds for I.

In four experiments in three animals similar results were obtained; in all except one experiment the sodium curve rose slightly ahead of the iodide curve and the rise time, duration, and rate of decline of the isotope concentration were all slightly faster for sodium than for iodide. In the remaining experiment sodium and iodide appeared at the same time. The mean appearance time

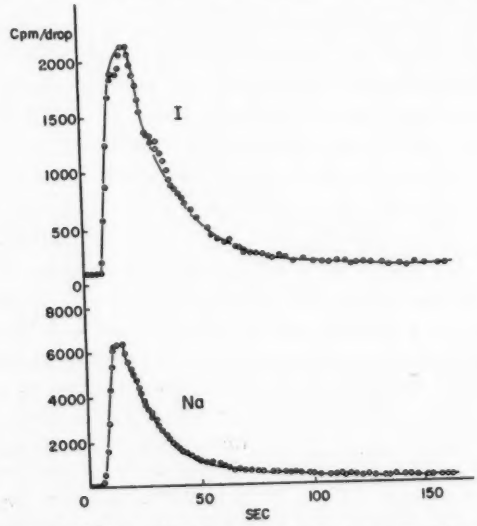

FIG. 1. Intra-arterial injection of 16 μc Na^{22} and 10 μc I^{131}. Mean rate of saliva secretion 0.314 ml/g/minute.

for sodium was 4.2 seconds and for iodide 4.9 seconds in the four experiments. There was never any inflection in the downstroke of the curve for either ion.

The appearance time for both isotopes is remarkly short when it is considered that this includes the circulation time to the gland (1–2 seconds) and the dead space in the salivary cannula and duct (1–2 seconds). The appearance time is, therefore, really not more than 2 to 3 seconds.

(b) Potassium (K^{42}) and Iodide (I^{131})

Figure 2 illustrates a typical experiment with potassium and iodide. In this experiment 12 μc of K^{42} and 1.5 μc of I^{131} were injected intra-arterially in less than 1 second. As far as the appearance and rise times are concerned potassium behaved in a similar manner to sodium. The appearance time was 7.5 seconds for potassium and 8.5 seconds for iodide and the time taken to reach 90% of the peak value was 8 seconds for both potassium and iodide. Potassium, however, differed strikingly from both sodium and iodide in the slowness with which the transient declined after its peak was past. In the period from 30 to 70 seconds both curves declined exponentially but whereas the rate constant for the iodide curve was 2.7 minute^{-1} ($t\frac{1}{2}$ = 15.3 seconds), that for potassium was 0.48 minute^{-1} ($t\frac{1}{2}$ = 86 seconds) less than a fifth of the rate for iodine. Three other experiments with potassium showed the same characteristics.

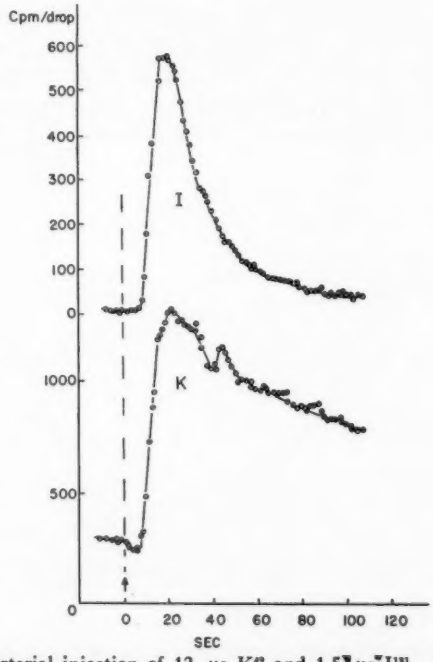

FIG. 2. Intra-arterial injection of 12 μc K^{42} and 1.5 μc I^{131}. Mean rate of saliva secretion 0.216 ml/g/minute.

Part II. Total Ion Concentration Transients

In these experiments, since equilibrium of specific activity between the isotope in the blood and gland had been established, the radioactivity in the saliva bears a constant proportionality to the total concentration of that ion.

(a) Iodide (I^{131})

Following a rest period there was a very pronounced iodide transient concentration. In the example shown in Fig. 3 the maximum level reached was eight times the level at 100 seconds when a nearly steady level had been attained. As we had found previously (2) the size of the iodide transient was dependent on the rest period—increasing in size with longer rest. The rise of the transient began very early after the start of secretion and reached 90% of its height in the 9 seconds following its take-off. In this instance the peak of the transient was a plateau lasting 4 to 5 seconds but the presence of this plateau was not invariable—often the peak of the iodide transient was brief as in Fig. 4. The transient (Fig. 3) declined exponentially towards the steady state with a half time of 14.3 seconds. In no case in a series of experiments did there appear to be any discontinuity in the falling phase of the iodide transient.

(b) Sodium (Na^{24})

Transients were present in all the curves obtained with sodium but they were quite small in magnitude compared with those seen with iodide. The sodium transients in the two examples shown (Figs. 4 and 5) represent increases of only 33% and 27% over the steady state whereas those for iodide represent 420% and 390%.

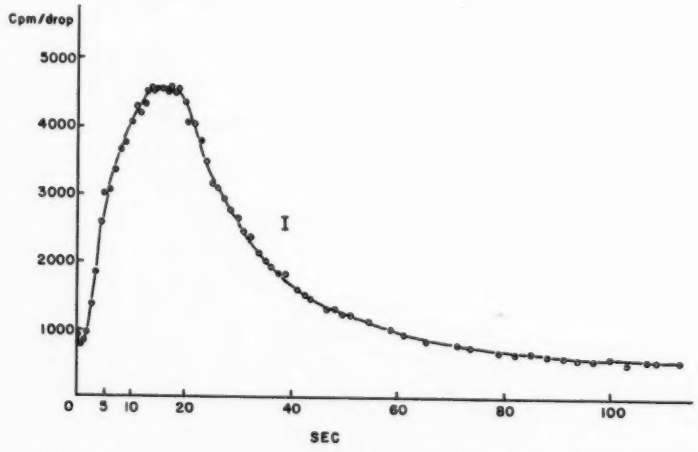

FIG. 3. Dog injected intravenously with NaI^{131} 3.3 μc/kg 20 minutes previously. Rest since last period of stimulation 15 minutes. Stimulation 20 c. Mean rate of saliva secretion 0.312 ml/g/minute.

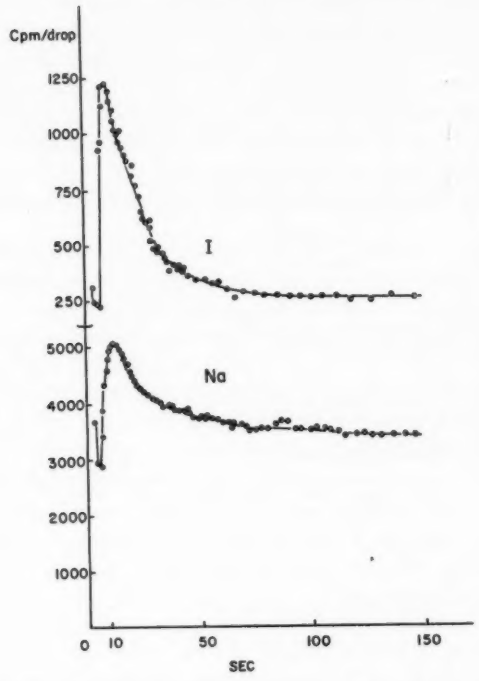

FIG. 4. Dog injected intravenously with Na^{24} 590 μc/kg and I^{131} 1.8 μc/kg. Rest since last period of stimulation 10 minutes. Stimulation 20 c. Mean rate of saliva secretion 0.284 ml/g/minute.

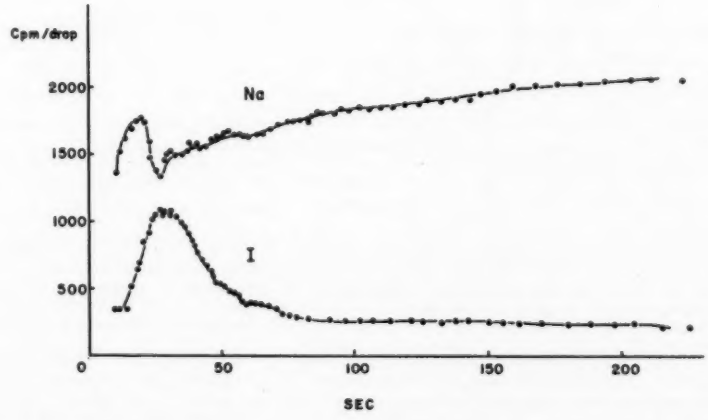

FIG. 5. Same dog as in Fig. 4. Rest period 10 minutes. Stimulation 6.3 c. Mean rate of saliva secretion 0.188 ml/g/minute.

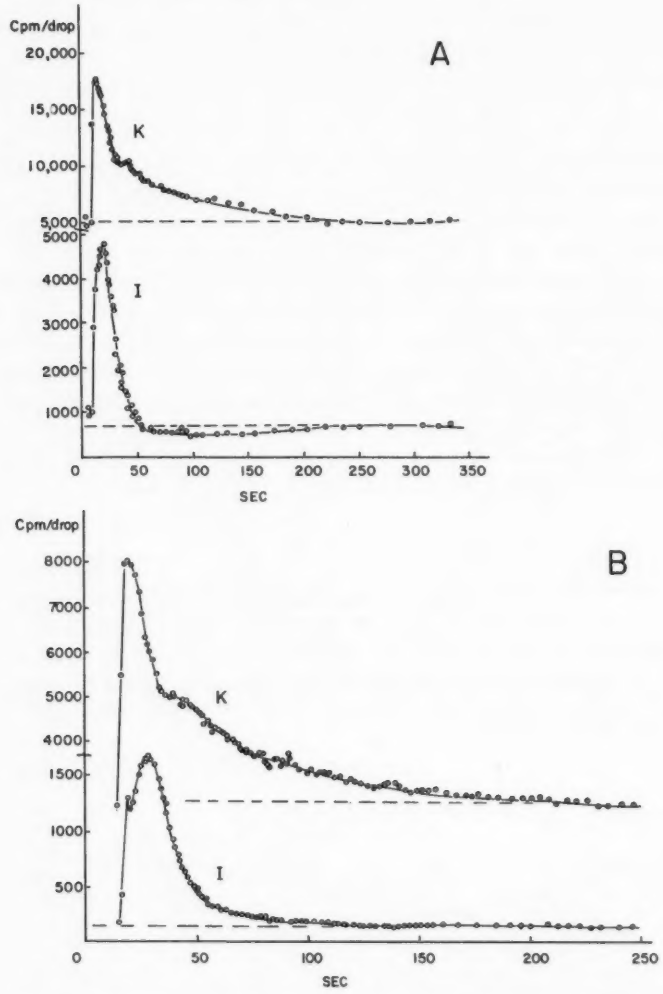

FIG. 6(*a*). Graded intravenous infusion of 10 mc K^{42} and 55 μc I^{131}. Rest since last period of stimulation 31 minutes. Stimulation 16 c. Mean rate of saliva secretion 0.220 ml/g/minute. Note the notch in the downstroke of the potassium curve that marks the intersection of the K_1 and K_2 transients. The iodide concentration is depressed during the period of the K_2 transient. The levels of the steady states are indicated by the broken lines.

(*b*). Graded intravenous infusion of 8 mc K^{42} and 55 μc I^{131}. Rest since last period of stimulation 19 minutes. Stimulation 20 c. Mean rate of saliva secretion 0.208 ml/g/minute. Note the inflection in the potassium curve at the intersection of the K_2 and K_1 transients. In this experiment there was no depression of the iodide level during the K_2 transient.

The other differences between the curves were that the sodium transient was always slightly earlier in time than the iodide transient and was shorter in duration. The late portions of the sodium curves were rarely parallel with the base line; this was due to the pronounced dependence of sodium concentration in saliva on the secretion rate which did not stay completely constant. The other two ions do not show such a dependence on flow rate. Insufficient data were obtained to give a reliable indication of the relationship between the magnitude of the sodium transient and the duration of the rest period.

(c) Potassium (K^{42})

In all experiments potassium showed a prominent transient which differed in several respects from those of sodium and iodide. The transient had a very short latency (Fig. 6) and always began ahead of the iodide transient. The recovery phase of the transient in three out of four satisfactory experiments showed a clear-cut inflection or discontinuity separating an early rapid recovery phase from a later slow recovery phase. While the first phase of the transient was both earlier and briefer than the iodide transient the second phase continued on for about 3 minutes.

Both phases of the potassium transient increased in size with greater rest intervals. In our experiments the maximum size of the first phase of the transient (K_1) was 3.6 times the steady state and the peak of the second phase (K_2) was 2.8 times the steady state. These correspond to potassium levels of 55 and 40 meq/l. respectively. In Fig. 6A may be seen another interesting phenomenon; following the iodide transient, the iodide level was depressed below that which it reached some 2 or 3 minutes later. This depression of iodide secretion, when it occurred, corresponded in time with the K_2 transient. It was not seen in all experiments (cf. Fig. 6B).

Discussion

The experiments with intra-arterial injections confirm previous findings that sodium, potassium, and iodide could readily cross the epithelium of the salivary ducts. In these experiments only duct transfer can account for the very short time elapsing between the intra-arterial injection and the appearance of the ions in the saliva. It has already been pointed out that after allowance has been made for circulation time and cannula dead space, the latency is of the order of 3 seconds at most. At a mean secretion rate of 250 μl/g minute this would correspond to only about 12 μl/g. Previously (2) reasons have been given for assuming that the duct capacity is 50–100 μl/g. The regions where ion exchanges occur must therefore extend quite distally in the duct system; sodium and potassium consistently appeared in the saliva drops slightly in advance of iodide, suggesting that the region of iodide transfer did not extend quite so distally as that for sodium and potassium. With potassium the situation was further complicated in that, while the rising limb of the isotope curve was not unlike that for the other two ions, the descending limb remained at a high level for some time after the peak, falling off about

five times more slowly than the curves for sodium and iodide. A slower disappearance of potassium from the blood could produce this effect, but since potassium disappears from the blood faster than either sodium or iodide, the prolongation of the potassium concentration in the saliva must be due to some property of the gland. In previous experiments (3) it was found that during activity the gland potassium exchanged with the blood with a rate constant of 0.1 to 0.4 per minute, a rate of the same order as the rate of decay of the potassium curve. This slow fall is therefore most probably due to release of potassium from a reservoir set up by the rapidly exchanging potassium of the cells. There is no direct evidence that the duct cells could not similarly establish a reservoir, but owing to their small volume such a reservoir would be too small to account for the long persistence of the curve. Further, if this reservoir were in the ducts, there would be a slow rise of the radioactive potassium concentration while the reservoir was filling as well as a slow fall while it emptied.

In spite of the rapid exchange of potassium between the acinar cells and blood the real contribution of cell potassium to the saliva is underestimated in these intra-arterial injection experiments, since in the very short time that K^{42}-laden blood is passing through the gland, equilibrium between cells and blood specific activity is not established. The fact that even in these circumstances, which minimize the cell contribution, there is a cellular potassium component in the saliva provides further evidence that a large part of the potassium, even in the steady state secretion, is derived from the acini. Figure 7 summarizes the evidence on the patterns of secretion obtained from intra-arterial injection experiments.

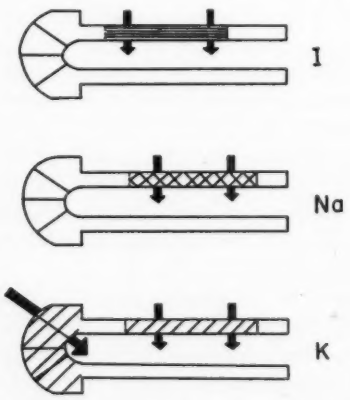

FIG. 7. Diagrammatic representation of the sites of transfer of ions from the intra-arterial experiments. Iodide, sodium, and potassium enter the saliva via the duct epithelium, the region of entrance of the latter two lying somewhat distal to that for iodide. Potassium also crosses the acinar cells in large amounts.

Even clearer evidence of two components in the potassium secretion came from the experiments in which the gland and blood were allowed to equilibrate with K^{42} than from the steady state experiments. The potassium transient started early after stimulation, synchronously with the sodium transient. Both preceded the iodide transient. The first part of the descending limb of the potassium transient curve terminated a phase whose duration and rate of fall were similar to those of sodium, and therefore clearly of duct origin (K_1). The end of this phase was marked by an inflection or hump on the declining limb of the potassium transient curve. The second phase (K_2) is associated with potassium of presumably acinar origin, and its characteristics have been discussed previously (1, 3).

The magnitude of the first phase (K_1) of the transient introduces a further complexity in the behavior of potassium. In two experiments the peak of the transient rose to 3.5 and 3.6 times the steady state level, corresponding to a potassium concentration in the saliva of 50 to 55 meq/l. Since this is about 10 times the concentration of potassium in blood, such a high concentration could not be produced by passive equilibration between isopotential phases of saliva and blood. If it were to be due to a passive equilibrium resulting from an electrochemical gradient, the potential difference between the duct lumen and the blood would have to be -55 to -60 mv. Such a potential difference seems improbable when it is compared with the value found by Lundberg (5) in the cat sublingual gland duct, where the lumen was a few millivolts positive to the duct exterior.

Since these high concentrations of potassium occur in the early saliva drops, representing secretion which had remained in the duct during the rest period, it might be suggested that potassium is concentrated by the osmotic reabsorption of water from the hypotonic saliva. But this cannot be the case; the osmotic gradient is insufficient to produce so great a concentration of potassium by osmotic water reabsorption in the ducts, unless it were augmented by sodium reabsorption as well, and sodium reabsorption in turn would be incompatible with a positive sodium transient. There may undoubtedly be a small osmotic component involved in this and other transients, but it must be inconsiderable in comparison with the much greater transfers found in these experiments. We are therefore led to conclude that at rest the duct epithelium can actively transfer potassium from the blood to the duct contents. This adds yet a further complexity in the behavior of potassium, since we now have evidence of its secretion into the saliva by the acinar cells, secretion by the resting duct cells, and net loss from the saliva across the duct cells during activity.

The iodide transient in the experiments after intravenous injection presents a few features that we have not considered previously. The transient began to rise after a latency of only 2 to 3 seconds, which suggests that the iodide-concentrating region extends more distally in the duct system than we had previously considered, but nevertheless not as distally as the limits of the region associated with the sodium and potassium transients whose latencies

are so short that their regions of transfer must extend even into the interlobular ducts. The relatively slow rise of the iodide curve, however, suggests that the more distal duct system is not as active in iodide transfer as is the intermediate region, a view consonant both with our previous findings in the dog (2) and with those of Logothetopoulos and Myant (4) in the hamster and the mouse. The flat top sometimes seen in the iodide transient may indicate a region of rather uniform concentrating capacity.

The asymmetry of the peak with a rather slow falling phase may be due to dispersion of the transient as the saliva flows out of the ducts, but it seems more probable that it is due to a reservoir of iodide in the duct cells which can empty into the lumen as the resting duct contents are expelled. A new finding of some interest was the depression of iodide level that sometimes followed the transient and was concurrent with a still raised potassium level in the saliva. This suggests that the activity of the iodide transport system may be sensitive to the level of potassium in the fluid bathing the lumenal face of the duct cells.

The sodium transient was relatively insignificant in magnitude and brief in duration. The present experiments leave it an open question whether this represents an active concentration of sodium by the resting ducts, a passive permittance of sodium diffusion along the blood–saliva concentration gradient, or a secondary concentration due to osmotic backflow of water from the saliva lying in the ducts. Further work will be needed to distinguish these alternatives.

The analysis of transients in the present work has shown up an error in a previous paper (1) in which no allowance was made for the delay in new acinar secretion reaching the exterior owing to the duct dead space. Recalculation of the data on potassium, sodium, and urea in the paper by Burgen and Seeman (1) leads to a reduction in the ratios of specific activity of saliva to cell content found. The values as recalculated show more uniform and lower values of these ratios which do not disturb but further emphasize the conclusions then reached, namely that extensive transductal movement of these substances must occur. In particular, the conclusion that a large part of the salivary sodium enters the saliva by crossing the duct epithelium is reinforced.

References

1. Burgen, A. S. V. and Seeman, P. Can. J. Biochem. and Physiol. **36**, 119 (1958).
2. Burgen, A. S. V. and Seeman, P. Can. J. Biochem. and Physiol. **35**, 481 (1957).
3. Burgen, A. S. V. J. Physiol. **132**, 20 (1956).
4. Logothetopoulos, J. H. and Myant, N. B. J. Physiol. **134**, 189 (1956).
5. Lundberg, A. Physiol. Revs. **38**, 21 (1958).

HISTAMINE RELEASE BY HOMOLOGOUS PLASMA IN THE DOG[1]

J. Q. Bliss and J. D. Walker

Abstract

Dogs were injected intracutaneously with their own plasma and with plasma from other dogs. The injected skin in each case was excised and incubated in Tyrode's medium. More free histamine was found in the media containing skin injected with homologous plasma than in the media containing skin injected with autologous plasma. When considered with earlier observations, this result justifies the conclusion that, in the dog, homologous plasma releases histamine on *initial* injection. Similar experiments with the histamine liberator compound 48/80 showed the method to be extremely sensitive in detecting histamine release.

Introduction

It can be shown with a simple experiment that a plasma incompatibility exists among dogs: intracutaneous injection of plasma from any other adult dog causes a wheal to form, while similar injection of the animal's own plasma is without effect (1, 2). The interesting aspects of this phenomenon include its remarkable degree of individual specificity (3), the frequency with which it is a factor in transfusion reactions (4), and the possibility that it is paralleled by a similar phenomenon in human subjects (2).

There is considerable evidence that in the dog homologous plasma produces its effects by releasing endogenous histamine. The whealing response to intracutaneous injection of plasma is inhibited by the antihistaminic mepyramine maleate (Neoantergan) and is reduced in areas of skin depleted of their endogenous histamine by previous exposure to a histamine liberator (2). Transfusions of homologous plasma are frequently followed by a marked increase in gastric acid secretion, and the urticarial reactions to such transfusions are abolished by mepyramine (4). Adrenalectomized dogs experience fatal anaphylactoid reactions to small transfusions of homologous plasma, and can be protected with the antihistaminic diphenhydramine hydrochloride (Benadryl) (5). Despite this evidence, conclusive proof of histamine release should rest on a demonstration of free histamine in tissue exposed to homologous plasma. Such a demonstration presents some difficulties, however, for the infrequency of hypotensive episodes in intact dogs following transfusion of homologous plasma (4) suggests that if histamine is released at all, it is released in relatively small amounts. With this in mind, we have developed a simple method for detecting the release of small amounts of histamine, and have used it to demonstrate histamine liberation by canine homologous plasma.

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Methods

Briefly, the method involves the intracutaneous injection of the suspected histamine liberator, followed by excision of the injected area and incubation in Tyrode's solution. Because of its high diffusibility and low affinity for tissue (6) some or all of any histamine released should distribute itself in the incubation medium. This medium is then assayed for free histamine.

Adult mongrel dogs of either sex were used. None of these animals had previously received any canine serum or other injections. At the start of each experiment, a pair of dogs was selected at random. One was designated as recipient, the other as donor. The recipient's plasma is referred to as *autologous* plasma, the donor's as *non-autologous* plasma. Blood was drawn by venipuncture, heparinized with 1 mg sodium heparin per 5 ml blood, and centrifuged at 3000 r.p.m. for 10 minutes ($r = 10$ cm). The recipient was then given intravenously 5 mg/kg of T-1824, a dye which is strongly bound by plasma protein (7); this procedure makes the identification of cutaneous wheals easier. Samples of autologous and non-autologous plasma serially diluted with isotonic saline were tested on the recipient as previously described (3). Where donor plasma failed to produce a distinct wheal at a 1:2 dilution, this donor was rejected and replaced. In 10 experiments this was necessary four times. These experiments therefore do not describe a strictly random sample, but a sample in which the degree of plasma incompatibility is somewhat more pronounced than in the general population.

After skin testing, the recipient dog was anaesthetized with sodium pentobarbital, 30 mg/kg, and a tracheotomy performed. One milliliter of non-autologous plasma was injected intracutaneously at a site chosen at random along the midaxillary line. When the first tinge of blue appeared at the site, the entire bleb raised by the injected plasma was rapidly undercut and excised with a sharp scalpel. The excised tissue consisted both of full-thickness skin and of 2–3 mm of subcutaneous tissue. This tissue was placed on filter paper, weighed, cut into slices approximately 2 mm thick, and dropped with the filter paper into a test tube containing 4.0 ml of Tyrode's solution. The entire procedure was then repeated with autologous plasma, using an injection site immediately adjacent to that of the non-autologous injection. The time interval between injection and excision was the same as that for non-autologous plasma. In each experiment, three pairs of injections were made, those of autologous plasma alternating with those of non-autologous plasma.

Each sample of excised, sliced tissue was allowed to incubate in Tyrode's solution at room temperature for 30 minutes. Then, following brief centrifugation, the supernate was pipetted off and treated with trichloroacetic acid to a final concentration of 10%. The resulting precipitate was removed by centrifugation, and 0.3 ml of concentrated hydrochloric acid was added to the supernate. This solution was held in a bath of boiling saline (107° C) for 90 minutes. The sample was then brought to pH 7.5 ± 0.5 with sodium hydroxide and 0.2 ml of 1 *M* tris(hydroxymethyl)aminomethane buffer, pH 7.7.

The neutralized samples were assayed for histamine on an isolated segment of guinea-pig ileum suspended in a 6-ml bath filled with Tyrode's solution (8)

containing 0.15 mg of atropine sulphate per liter. No more than 0.1 ml of sample was added to the bath at a time, for larger additions had a depressant effect on the gut, probably owing to their hypertonicity. Because the amount of histamine in the samples often was small, guts insensitive to 0.003 μg were rejected and replaced. At the end of a series of assays, 0.020 μg of mepyramine maleate was added to the bath. After several washings, some assays were repeated to test parallel inhibition of the histamine standard and samples. These tests never indicated a contracting substance other than histamine.

To ensure the validity of the method, the recovery of standard histamine solutions treated in the same way as supernates from the incubated tissue was tested. In eight trials, the mean recovery was 87 ± 7 (S.D.)%.

The amount of free histamine recovered per sample of skin, in μg, was calculated from the expression $(A/V) \times (4+S)$, where A = total histamine in assayed sample, in μg, V = volume of supernate removed after incubation, in ml, and S = weight of excised tissue, in g ($4+S$ represents fairly closely the original volume through which any free histamine can distribute itself).

All values for histamine are expressed as the base.

The histamine liberator compound 48/80 (9), dissolved in isotonic saline, was tested in the same way as plasma in some experiments. At the end of these experiments, the minimum dose of 48/80 which would produce a 10% fall in arterial blood pressure was determined. Blood pressure was recorded with a mercury manometer from a cannulated femoral artery; 48/80 was given intravenously in ascending doses at 5-minute intervals.

Results

The results of 10 consecutive experiments are shown in Table I. The amount of free histamine recovered after non-autologous plasma injection was on the average 0.296 ± 0.029 (S.E.) μg, after autologous plasma 0.141 ± 0.013 μg. Application of the t test to individually paired values showed this difference to be significant ($P < 0.001$).

TABLE I

Free histamine recovered after plasma injection

	Non-autologous plasma, μg		Autologous plasma, μg	
Expt. No.	Mean	S.D.	Mean	S.D.
1	0.49	± 0.11	0.15	± 0.05
2	0.55	0.11	0.30	0.02
3	0.33	0.06	0.14	0.05
4	0.26	0.09	0.14	0.08
5	0.25	0.02	0.06	0.02
6	0.15	0.05	0.06	0.03
7	0.12	0.02	0.12	0.03
8	0.15	0.02	0.15	0.01
9	0.21	0.02	0.11	0.01
10	0.45	0.08	0.18	0.04

Two possible variables besides the source of the plasma might account for this difference: the amount of tissue excised (to which the amount of spontaneous histamine release is related), and the presence of small quantities of extravascular T-1824 in the tissue treated with non-autologous plasma. The weight of tissue excised after non-autologous injection was on the average 1.43 g, after autologous injection 1.38 g. This difference is not significant (S.E. of the difference $=0.05$ g, $P>0.2$), nor is it in any case large enough to be of importance. In experiments 9 and 10, T-1824 was not given, and tissue was excised after an interval arbitrarily set at $2\frac{1}{2}$ minutes after injection; the difference in histamine release was as marked as in the other experiments.

Results obtained with compound 48/80, in the two experiments in which it was used, are shown in Table II. The skin injection method was sensitive to 1/200 to 1/500 of the dose of 48/80 needed to cause an appreciable fall in blood pressure.

TABLE II

Free histamine recovered after 1.0-ml injections of compound 48/80 and autologous plasma

Concn. of 48/80, g/ml	Expt. No. 6		Expt. No. 7	
	48/80, μg	Plasma, μg (adjacent area)	48/80, μg	Plasma, μg (adjacent area)
10^{-3}	0.23	0.09	0.43	0.10
10^{-4}	0.18	0.05	0.30	0.10
10^{-5}	0.16	0.04	0.23	0.15
10^{-6}	0.12	0.04	0.22	
10^{-7}	0.04		0.13	
Dose of 48/80 causing 10% fall in B.P.	0.2 mg (0.025 mg/kg)		0.5 mg (0.025 mg/kg)	

Discussion

These experiments show that in the dog more free histamine is recovered from skin injected with non-autologous plasma than from skin injected with autologous plasma. This observation, when considered with earlier results (2, 4, 5) described in the Introduction, justifies the conclusion that homologous plasma produces most or all of its effects in the dog by releasing endogenous histamine. The implications of this situation have been discussed previously (2, 3, 4).

The recovery of some free histamine from skin injected with autologous plasma should not be interpreted as an actual release of histamine by such plasma. It is much more likely that this free histamine is a product of spontaneous release and the trauma of excision and slicing, for intracutaneous or intravenous injection of autologous plasma rarely if ever causes any untoward reaction (2, 3, 4).

Although the method used here gives a good qualitative assessment of potential histamine liberators, it needs further development before it can be used for a strictly quantitative measure of the total amount of histamine

released. Some liberated histamine is probably removed by the circulation before excision of the injected tissue, and there is no good assurance that free histamine in the removed tissue reaches complete equilibrium with the incubation medium. In the present experiments, however, there is some reason for believing that non-autologous plasma releases only a small portion of the total skin histamine. The amount of release as estimated by our method is small (<10%) relative to the total skin histamine content of dogs (10, 11); in a few pilot experiments we were unable to find differences in the residual skin histamine after injection of autologous and non-autologous plasma; and in some unpublished experiments we have found that compound 48/80 is still able to produce marked whealing in areas of skin previously injected with non-autologous plasma.

The method described here seems to be well suited for the detection of histamine liberators of low potency, or of higher potency but available only in minute amounts. It detected histamine release by as little as 1 μg of 48/80, a dose several hundred times less than the intravenous dose needed to cause hypotension. It has the further advantage of permitting the screening in one animal of many drugs for histamine-releasing properties.

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References

1. Freeman, N. E. and Schecter, A. E. Science, **96**, 39 (1942).
2. Bliss, J. Q. and Stewart, P. B. Can. Med. Assoc. J. **76**, 847 (1957).
3. Bliss, J. Q., Stewart, P. B., and Fuller, J. L. Brit. J. Exptl. Pathol. **39**, 30 (1958).
4. Bliss, J. Q., Johns, D. G., and Burgen, A. S. V. Circulation Research. In press.
5. Baker, C. H. and Remington, J. W. Circulation Research, **6**, 294 (1958).
6. Miles, A. A. and Miles, E. M. J. Physiol. (London), **118**, 228 (1952).
7. Rawson, R. A. Am. J. Physiol. **138**, 708 (1943).
8. Code, C. F. and McIntire, F. C. Methods of Biochem. Anal. **3**, 66 (1956).
9. Paton, W. D. M. Brit. J. Pharmacol. **6**, 499 (1951).
10. Feldberg, W. *In* Ciba Foundation symposium on histamine. J. & A. Churchill, Ltd., London. 1956. p. 6.
11. Perry, W. L. M. *In* Ciba Foundation symposium on histamine. J. & A. Churchill, Ltd., London. 1956. p. 245.

STRUCTURAL STUDIES ON CLINICAL DEXTRANS

PART 1. METHYLATION AND PERIODATE OXIDATION STUDIES[1]

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Abstract

Determinations have been made of the structures of four clinical dextrans, i.e. partially depolymerized dextrans which, in solution, are used as blood-volume expanders. By methylation studies, the major fractions of the dextrans have been shown to consist of chains of α 1→6 linked glucopyranose residues about 1 in every 9.5 – 11 of which also have branch points at C_3. Two of the dextrans are probably also branched to a minor extent on C_4. Periodate oxidation studies are reported on the clinical dextrans and on fractions obtained by ultrafiltration.

Dextran, a polysaccharide containing glucopyranose residues, is used on a manufacturing scale for the production of solutions of blood-volume expanders (12, 26). The most commonly employed dextran is that produced by *Leuconostoc mesenteroides* strain NRRL B-512 and substrain B-512F. Industrially, the polysaccharides are partially depolymerized by acidic or enzymatic hydrolysis or by thermal degradation. The material in the neutral, or neutralized, hydrolyzate is then fractionated by the addition of acetone or ethanol to obtain material of a molecular weight distribution deemed suitable for infusion into humans. The specifications for clinical dextrans vary from country to country, and the various manufacturers use procedures which differ in certain details, most notably in the method used to effect depolymerization.

Clinical dextrans would be expected to exhibit certain, possibly all, of the structural details present in the parent dextran. If, however, there were any structural elements in the parent dextran that were labile under the conditions used to effect depolymerization, they would be absent, or present in a disproportionately low amount, in the clinical material. It is evident, therefore, that a knowledge of the structure of the clinical dextran will contribute to a knowledge of the parent native dextran and vice versa. It cannot be discounted that modifications, other than those due to simple hydrolysis, may have taken place in those cases where acidic (6*a*) or enzymatic hydrolysis or thermal degradation (11*a*) was used to effect depolymerization. Neutralization and other procedures which resulted in the temporary or permanent production of alkaline conditions at any stage in the manufacture might result in a modification of the reducing end groups of the dextran molecules (7, 20). Such a modification was adduced to have taken place in certain cases. Bixler *et al.* (4) reported that, in an industrial

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process used for the production of clinical dextran, strong sodium hydroxide was employed to reduce acidity and a more dilute solution to achieve neutrality: such conditions may favor end-group modification.

Although there is a vast literature on clinical dextrans (17), little has been published on their structures. In the present work four clinical dextrans, derived from native dextrans produced by *L. mesenteroides*, have been examined; they were manufactured in Canada, England, Sweden, and the United States, and are subsequently called C-, E-, S-, and US-dextrans. Weissberg (31), on the basis of physicochemical measurements on commercial clinical dextrans, concluded that the E-dextran had a higher degree of branching than had the US-dextran.

Most of the work on dextrans has been on the native polymer. Comparatively slight modifications of culture conditions may cause a marked difference in the fine structure of the dextran produced. Barker *et al.* (2) noted that a dextran produced by *Betacoccus arabinosaceous* (Birmingham strain), grown on a medium deficient in magnesium, was very much less branched than was one produced in an environment relatively richer in magnesium (1, 3). Stacey and Swift (27) by methylation studies determined that the dextran produced by this latter microorganism consisted of α-1,6-linked glucopyranose residues with side chains of similarly linked residues attached to C_4 positions. Some 5 years later Barker *et al.* (3) determined that the side chains on a dextran produced by a substrain of the same microorganism were attached to C_3 not to C_4. This discrepancy may have been due to a misinterpretation of the earlier findings, but it is possible that a strain selection or a mutation may have taken place due to subculturing (3) during the intervening years. Fowler *et al.* (10) concluded that the dextran produced by *L. mesenteroides* possessed a large number of branch points on C_4 but this work was criticized by Brauns (5).

Much work on the structure of dextrans has been carried out by Jeanes *et al.* (18, 19, 24, 25). The conclusion that a dextran produced by *L. mesenteroides* (NRRL B-512) consisted of 95% 1,6-linked glucopyranose residues, was confirmed by Van Cleve *et al.* (29) but, whereas the periodate oxidation studies (18) indicated that *ca.* 5% of those residues carried side chains on C_4, Van Cleve by methylation studies showed that they were actually attached to C_3. A similar anomaly was observed in the present work.

Experimental

Four clinical dextrans were investigated. They were supplied in the form in which they are used as blood-volume expanders, that is as *ca.* 6% solutions of clinical dextran in 0.9% saline. The solutions had been sterilized and vacuum-sealed in bottles by the manufacturers. The specifications of the clinical dextrans varied according to their country of origin and were those pertaining prior to 1955. The clinical dextrans are commercially known as

Hydrolysed Dextran,[3] Intradex,[4] Macrodex (or Pharmadex),[5] and Expandex[6]. A sample of Intradex-type clinical dextran was also supplied in powder form, not under vacuum seal, and this was used in ultrafiltration studies (32). All the dextrans are known to have been produced by *L. mesenteroides* NRRL B-512 or B-512F. The S- and US-dextrans had been produced by acidic hydrolysis of the native dextran, the C-dextran by enzymatic degradation, and the E-dextran by thermal degradation.

Throughout the remainder of the paper the term dextran is used in place of clinical dextran.

General Methods

Paper chromatograms (Whatman Nos. 1 and 3MM) were irrigated by the descending method. All solvents and irrigants used in quantitative separations were of high purity. The following irrigants were used (where there were two layers, the top one was taken): A, benzene–ethanol–water (169:47:15 v/v); B, butan-1-ol–benzene–pyridine–water (5:3:3:1 v/v); C, ethyl acetate – acetic acid – formic acid – water (18:3:1:4 v/v); D, butan-1-ol–pyridine–water (10:3:3 v/v); E, butan-1-ol–pyridine–water (100:33:50 v/v); F, methyl ethyl ketone saturated with water; G, butan-1-ol–ethanol–water (10:3:5 v/v).

Sugars were detected on chromatograms by the use of either (*a*) *p*-anisidine hydrochloride in butanol (14) or (*b*) ethanolic silver nitrate in acetone and sodium hydroxide (23). Where possible, authentic samples were chromatographed on the same paper as the unknown. The R_g value of a sugar is its rate of movement on a chromatogram relative to that of 2,3,4,6-tetra-*O*-methyl-D-glucose.

Solutions were either concentrated under reduced pressure at the lowest possible temperature or were lyophilized. Ashes were sulphated but no corrections were made for ash in any of the calculations. Methoxyl determinations were made by the method of Vieböck and Brecker (30) with certain modifications due to Easterbrook and Hamilton (8).

Isolation of Salt-Free Dextran

Where the dextrans were required salt-free, they were dialyzed against running tap water until free from chloride ions; a trace of mercuric chloride was added to the dialysis bag from time to time to act as a bactericide. During dialysis of the C-dextran a slight precipitate settled inside the bag. The precipitate was separated and treated with 2 *N* hydrochloric acid at 100° for 8 hours, and the mixture neutralized with silver carbonate. A concentrate of the solution was examined chromatographically (irrigant C). No

[3]Hydrolysed dextran (C-dextran): Lot 76-1. Supplied by the Connaught Laboratories, Dufferin Division, University of Toronto, Toronto, Ontario.

[4]Intradex (E-dextran): Lot 54/013 (manufactured 1954) in solution, and Lot 54/091 in powder form. Supplied by Glaxo (Canada) Ltd., Saxony Buildings, Duncan Street, Toronto, Ontario, and by Glaxo Ltd., Barnard Castle, Co. Durham, England.

[5]Macrodex or Pharmadex (S-dextran): Batch D-214. Supplied by Pharmacia Ltd., Uppsala, Sweden.

[6]Expandex (US-dextran): Lot 285W3-A. Supplied by Commercial Solvents Corporation, Terre Haute, Indiana, U.S.A.

spots were observed on papers sprayed with spray (*a*) but, on a paper sprayed with 0.15% ninhydrin in chloroform two streaks were observed, one blue (R_F, 0–0.22) and the other grey (R_F, 0.29–0.66). No nitrogen was detected when the precipitate was examined. The dextran solution from another bottle of C-dextran did not give rise to any precipitate under similar circumstances.

The dextrans were precipitated by adding them in *ca.* 1% aqueous solution to 3 to 4 times their volume of vigorously stirred ethanol. The dextrans separated as white powders which were washed, by decantation, several times with ethanol, then with ether, and were dried under reduced pressure. The use of a solution more concentrated than 1% resulted in the formation of a viscous syrup which was unsuitable for quantitative work and was difficult, or impossible, to dissolve when dry.

Other samples of the dextran solutions were dialyzed for 10 days against static distilled water containing a trace of mercuric chloride as bactericide. The solutions were then concentrated and Molisch tests carried out on each concentrate. The dialyzable material from the C-dextran gave a much stronger test than did that from any of the other dextrans. Chromatographic examination of the solutions indicated the presence of small amounts of higher oligosaccharides in all of the solutions; there were no indications of the presence of fructose.

Methylation of the Dextrans

All samples of precipitated, but not salt-free, dextran were treated for a period of 2 to 5 weeks with solutions of diazomethane in moist ether in order to protect the reducing end groups from subsequent alkaline oxidation. The various products were then methylated as follows.

C-Dextran

The sample (10 g) was treated with 30% aqueous sodium hydroxide (10 × 125 ml) and methyl sulphate (10×90 ml). The reagents were added dropwise and together and initial additions were made in an atmosphere of oxygen-free nitrogen. In later additions acetone was added from time to time to increase the solubility of the by then partially methylated and partly precipitated dextran. After neutralization of the reaction mixture with sulphuric acid, the methylated product was separated. Yield, 7.8 g. It was a grey friable solid having OCH_3, 40.6% (theoretical for $C_9H_{16}O_5$, 45.6%). An attempt was made to methylate the product further by treating it, in dioxan, with the above reagents. After six treatments the product had OCH_3, 41.1%. This product (7.4 g) was dissolved in liquid ammonia (*ca.* 60 ml) and treated with sodium (1.5 g) and methyl iodide (3 ml) by a modification (3, 11) of the method of Muskat (22). The ammonia was allowed to evaporate overnight and five other similar treatments were given. The product was washed with warm water and dried. It was a dark grey powder readily soluble in chloroform even when wet. Yield, 6.0 g (60% of starting

material). It had OCH_3, 44.1%; sulphated ash, 0.21%. It was dissolved in chloroform and precipitated by the addition of an excess of light petroleum (b.p. 80–100°). The dry product had $[\alpha]_D^{23}$, + 199° (*c*, 1 in $CHCl_3$).

E-Dextran

A sample (10 g) was treated in essentially the same way as was the C-dextran but omitting the methylations in dioxan. The product was dissolved in petroleum ether and reprecipitated as before. The brown product had OCH_3, 44.7%; sulphated ash, 0.12%; and $[\alpha]_D^{21}$, + 198° (*c*, 1 in $CHCl_3$). Yield, 6.30 g (63% of starting material).

S-Dextran

A sample (10 g) was treated with methyl sulphate and sodium hydroxide. After 16 treatments it had OCH_3, 39.0%. After treatment by the modified Muskat procedure the reprecipitated material had OCH_3, 44.6%; sulphated ash, 0.31%; and $[\alpha]_D^{23}$ +207° (*c*, 1 in $CHCl_3$). Yield, 7.5 g (75% of starting material).

US-Dextran

A sample (9 g) was treated in the same way as the S-dextran. After reprecipitation of the methylated product it had OCH_3, 44.4%; sulphated ash, 0.18%; and $[\alpha]_D^{23}$, + 205° (*c*, 1 in $CHCl_3$). Yield, 5.2 g (58% of starting material).

TABLE I

Methylated dextran	Fraction	B.p. light petroleum	Ratio of light petroleum to chloroform	Weight, g	OCH_3, %	Appearance of syrup*
C-Dextran	1	35–48°	6:1	0.027	—	br
	2	35–48°	4:1	0.39	—	c
	3	60–80°	4:1	4.30	45.2	p br
	4	60–80°	3:1	0.98	—	p br
	5	60–80°	2:1	0.20	—	dk br
	Residue	—	—	0.02	—	dk br
E-Dextran	1	66–75°	5:1	0.12	—	p br
	2	66–75°	4:1	3.56	45.3	p br
	3	66–75°	3:1	1.88	—	br
	4	66–75°	5:2	0.40	—	dk br
	5	66–75°	4:3	0.027	—	—
	Residue	—	—	0.00	—	—
S-Dextran	1	66–75°	5:1	0.12	—	dk br
	2	66–75°	4:1	5.21	45.3	p br
	3	66–75°	3:1	1.30	44.3	br
	4	66–75°	5:2	0.52	43.0	dk br
	Residue	—	—	0.23	—	dk br
US-Dextran	1	60–80°	5:1	0.03	—	c
	2	60–80°	4:1	0.29	—	p br
	3	60–80°	3:1	3.12	45.0	p br
	4	60–80°	5:2	1.30	—	br
	Residue	—	—	0.035	—	—

*br—brown, c—colorless, dk—dark, p—pale.

Fractionation of Methylated Dextrans

The methylated dextrans were fractionated by dissolution in successive volumes (300–500 ml) of mixtures of light petroleum and chloroform progressively enriched with respect to the second component. After each treatment the solution was decanted and the solvents were distilled off (see Table I).

Hydrolysis of the Methylated Dextran Fractions

Samples of five of the methylated dextran fractions (C-3, 4.10 g; E-2, 2.02 g; S-2, 2.2 g; S-3, 1.15 g; and US-3, 2.75 g) were dissolved in 90% formic acid, *ca.* 200 ml/g methylated dextran. When dissolution was complete the solution was allowed to stand at room temperature for about 12 hours, then the temperature was raised to 60° for 3 hours and finally to 90° for 24 to 36 hours. The optical rotation of the C-3 fraction, 4.10 g in *ca.* 800 ml 90% formic acid, was followed from the time at which heating was started:

Hours	0	0.6	4	12	24.5	36
α^{25}_{obs}	0.64	0.56	0.37	0.31	0.24	0.24

The formic acid was removed under reduced pressure, water being added at frequent intervals. The formyl esters were hydrolyzed by being heated in aqueous solution (*ca.* 125 ml water/g methylated dextran) for 6 hours. The water and formic acid were distilled off. This procedure was repeated a further three times with 6-hour heating periods and once with heating for 10 hours. The solutions obtained were either pale yellow or pale brown but when, in two experiments, 98% instead of 90% formic acid was used, extensive charring took place. The hydrolyzate mixtures were much darker in color if, when 90% formic acid was used, the initial period during which the mixture was allowed to stand at room temperature after complete dissolution of the methylated dextran was omitted and heating was begun immediately. A sample of the formic acid hydrolyzed product from C-3 was treated with *N* sulphuric acid (100°, 6 hours) and the neutralized hydrolyzate was examined on chromatograms (irrigants B, C, and G) along with a sample of the autohydrolyzed product obtained on the above treatment with water. No difference was observed in the number, type, or proportion of the various components in the two samples.

Fractionation of the Hydrolyzates

The hydrolyzed fractions of C-3, E-2, S-2, and US-3 were separated chromatographically on cellulose columns (4×45 cm) irrigated with a mixture (4:1) of benzene–ethanol–water (169:47:15 v/v top layer) and of benzene–ethanol (169:47 v/v). After the trimethyl fractions had been eluted the irrigant was changed to ethyl acetate – acetic acid – formic acid – water (18:3:1:4 v/v).

The hydrolyzate of fraction S-3 was concentrated to a syrup and a portion of it was chromatographically fractionated (irrigant A; Whatman No. 3MM paper). The positions of the various sugars were located in the usual way and appropriate areas of paper were cut out to contain only one sugar each. The components were extracted with hot water and hot methanol.

Examination of the Fractionated Hydrolyzates

Hydrolyzate of C-3

Fraction 1 (270 mg) was chromatographically indistinguishable from 2,3,4,6-tetra-*O*-methyl-D-glucose. On standing at 0° the syrup crystallized. The product had m.p. and mixed m.p. 82–84°, $[\alpha]_D^{25}$ + 86° (*c*, 0.3 in water).

A sample (20 mg) was heated at 80° with a 2% solution of freshly distilled aniline in ethanol. On removal of the solvent, crystals separated and were recrystallized from a mixture of light petroleum (b.p. 60–80°) and ether. The product had m.p. 135–136° (lit. 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-glucosylamine, m.p. 137–138°), $[\alpha]_D^{23}$ +231 (*c*, 0.15 in acetone).

Fraction 2 (2.21 g) was indistinguishable on chromatograms from 2,3,4-tri-*O*-methyl-D-glucose and contained a trace of a component having the same R_g value (irrigants A and D) as 2,4-di-*O*-methyl-D-glucose. The syrup did not crystallize. The anilide was prepared and after recrystallization had m.p. 145–146° (lit. 2,3,4-tri-*O*-methyl-*N*-phenyl-D-glucosylamine, m.p. 147–148°).

Fraction 3 (119 mg) contained two components. One was chromatographically indistinguishable from 2,3,4-tri-*O*-methyl-D-glucose and the other chromatographically (irrigants A, C, D, E, and F) and electrophoretically (0.5 *M* borate in 0.1 *N* sodium hydroxide) distinguishable from 2,3-, and 3,4-di-*O*-methyl-D-glucose but indistinguishable from 2,4-di-*O*-methyl-D-glucose. Visual estimation of the relative proportion of the two components, based on the relative intensities and areas of the spots produced on chromatograms, indicated the ratio of the tri- to di-*O*-methyl glucose to be 1:5.

Fraction 4 (116 mg) possessed identical chromatographic and electrophoretic properties to the principal component in fraction 3. The syrup crystallized on seeding with a crystal of 2,4-di-*O*-methyl-D-glucose. The crystals had m.p. and mixed m.p. 124–128°.

The fraction was treated with *p*-nitroaniline in ethanol in the presence of a trace of acetic acid (28) and on standing yielded crystals which had m.p. 247–250° (lit., *N*-*p*-nitrophenyl-2,4-di-*O*-methyl-D-glucosylamine, m.p. 250–251°).

Fraction 5 (37 mg) was a syrup which did not crystallize. Chromatographic examination showed it to be indistinguishable from 2,3-di-*O*-methyl-D-glucose. An attempt was made to prepare the anilide but no crystalline product was obtained.

Summary of weights of components in hydrolyzate.—2,3,4,6-Tetra-*O*-methyl-D-glucose: fraction 1, 270 mg. 2,3,4,-Tri-*O*-methyl-D-glucose: fraction 2, 2.21 g; fraction 3 *ca.* 0.02 g; total weight, 2.23 g. 2,4-Di-*O*-methyl-D-glucose: fraction 3, *ca.* 99 mg; fraction 4, 116 mg; total weight, 215 mg. (?,2,3)-Di-*O*-methyl-D-glucose: fraction 5, 37 mg.

Molar ratios of residues in C-3 dextran.—2,3,4,6-Tetra-, 2,3,4-tri-, 2,4-di-, (?,2,3)-di-*O*-methyl-D-glucose were present in the molar ratios of 1:8.8:0.92:0.16.

Hydrolyzate of E-2

The components present in the various fractions possessed chromatographic properties identical with those exhibited by the fractions in the previous methylated dextran hydrolyzate.

Fraction 1 (179 mg) was a brown syrup which crystallized. On recrystallization it had m.p. 85–86° and $[\alpha]_D^{25}$ +84° (*c*, 1.0 in water). The anilide was prepared. The recrystallized product had m.p. 135–136° and this was not depressed on admixture with crystals of the anilide of C-3 fraction 1.

Fraction 2 (1.27 g) was a syrup from which the anilide was prepared. The crystalline product had m.p. 144–146° and did not depress the m.p. of 2,3,4-tri-*O*-methyl-D-glucosylamine.

Fraction 3 (77 mg) was a mixture of two components chromatographically indistinguishable from 2,3,4-tri-*O*-methyl-D-glucose and 2,4-di-*O*-methyl-D-glucose. The molar ratio of the two components was estimated, by the method given above, to be 1:7. The syrup did not crystallize.

Fraction 4 (112 mg) was a mixture of two components. One of them was chromatographically indistinguishable from 2,3-di-*O*-methyl-D-glucose. They were present in the ratio of *ca.* 10:1. A portion of the syrup was fractionated on Whatman No. 3MM paper (irrigant A) and 25 mg of the faster component isolated. The *p*-nitroanilide was prepared and had m.p. 245–248° not depressed on admixture with the corresponding derivative of fraction 4 of the C-3 hydrolyzate. It had $[\alpha]_D^{20}$ −262° (*c*, 0.1 in pyridine).

Fraction 5 (22 mg) contained only one component. It was chromatographically indistinguishable from 2,3-di-*O*-methyl-D-glucose. The anilide was prepared and after recrystallization from light petroleum had m.p. 134°. Yield of crystalline material 21 mg.

Summary of weights of components in hydrolyzate.—2,3,4,6-Tetra-*O*-methyl-D-glucose: fraction 1, 179 mg. 2,3,4-Tri-*O*-methyl-D-glucose: fraction 2, 1.27 g; fraction 3, *ca.* 0.010 g; total weight, 1.28 g. 2,4-Di-*O*-methyl-D-glucose: fraction 3, *ca.* 68 mg; fraction 4, 112 mg; total weight, 180 mg. 2,3-Di-*O*-methyl-D-glucose: fraction 5, 22 mg.

Molar ratios of residues in E-2 dextran.—2,3,4,6-Tetra-, 2,3,4-tri-, 2,4-di-, and 2,3-di-*O*-methyl-D-glucose were present in the molar ratios of 1:7.6:1.14:0.14.

Hydrolyzate of S-2

The components present in the various fractions possessed chromatographic properties indistinguishable from those of the fractions from the C-3 hydrolyzate but, in addition, another component, believed to be 3,4-di-*O*-methyl-D-glucose and possibly a very faint trace of an acidic-type component were noted.

Fraction 1 (198 mg) crystallized. The crystals had m.p. 82–83° and $[\alpha]_D^{23}$ +84° (*c*, 0.5 in water).

Fraction 2 (1.62 g) was a brown syrup from which the anilide was prepared. The latter on recrystallization had m.p. 145–146°.

Fraction 3 (85 mg) contained two components, one chromatographically indistinguishable from 2,3,4-tri-*O*-methyl-D-glucose, and the other from 2,4-di-*O*-methyl-D-glucose. They were present in a ratio of 1:13.

Fraction 4 (138 mg) contained a major and a trace component which were chromatographically indistinguishable from 2,4-di-*O*-methyl-D-glucose and 2,3-di-*O*-methyl-D-glucose respectively. A faint trace of a component was detected on chromatograms run in irrigant E; the component had R_g 0.22. The mixture was fractionated (irrigant A) on Whatman 3MM paper and 40 mg of a chromatographically homogeneous syrup was obtained which, on seeding with 2,4-di-*O*-methyl-D-glucose, slowly crystallized (m.p. 124–127°).

Fraction 5 (30 mg) contained two components. One was chromatographically indistinguishable from 2,3-di-*O*-methyl-D-glucose and the other was distinguishable from 2,4-di-*O*-methyl-D-glucose (irrigants A, C, and E).

Summary of weights of components in hydrolyzate.—2,3,4,6-Tetra-*O*-methyl-D-glucose: fraction 1, 198 mg. 2,3,4-Tri-*O*-methyl-D-glucose: fraction 2, 1.62 g; fraction 3, 0.02 g; Total weight, 1.64 g. 2,4-Di-*O*-methyl-D-glucose: fraction 3, *ca.* 64 mg; fraction 4, *ca.* 138 mg; total weight, *ca.* 202 mg. Di-*O*-methyl-D-glucose: fraction 5, 30 mg.

Molar ratios of residues in S-2 dextran.—2,3,4,6-Tetra-, 2,3,4-tri-, 2,4-di-, 2,3-di-, + (3,4?)-di-*O*-methyl-D-glucose were present in the molar ratios of 1:8.8:1.16:0.17 + a trace of an unidentified acidic-type component.

Hydrolyzate of S-3

No attempt was made to prepare derivatives, only the weights of the fractions were determined and the fractions correlated to the corresponding fraction in the hydrolyzate of S-2 by chromatographic and electrophoretic examination.

Summary of the weights of components in hydrolyzate.—2,3,4,6-Tetra-*O*-methyl-D-glucose: 0.068 g. 2,3,4-Tri-*O*-methyl-D-glucose: 0.322 g. 2,4-Di-*O*-methyl-D-glucose: 0.071 g. Other di-*O*-methyl-D-glucoses: 0.019 g.

Molar ratios of residues in S-3 dextran.—2,3,4,6-Tetra-, 2,3,4-tri-, 2,4-di-, and other di-*O*-methyl-D-glucoses were present in the molar ratios of 1:5.0:1.2:0.32. A trace of a component was detected which was chromatographically indistinguishable from the acidic-type component detected in the S-2 hydrolyzate.

Hydrolyzate of US-3

The hydrolyzate contained components which were indistinguishable from those found in the S-2 hydrolyzate. The acidic-type component was present in greater, although still trace, amount.

Fraction 1 (22.0 mg) was a syrup which crystallized on seeding with 2,3,4,6-tetra-*O*-methyl-D-glucose. The recrystallized fraction had m.p. 82–83° and $[\alpha]_D^{25}$ 82.5° (*c*, 0.1 in water).

Fraction 2 (190 mg) was a syrup which contained a trace of 2,3,4,6-tetra-*O*-methyl-D-glucose. The anilide was prepared and crystallized on seeding

with 2,3,4-tri-*O*-methyl-*N*-phenyl-D-glucosylamine. The product had m.p. and mixed m.p. 145–147°.

Fraction 3 (23.5 mg) was chromatographically indistinguishable from 2,4-di-*O*-methyl-D-glucose. The *p*-nitroanilide derivative was prepared: m.p. 248–250°.

Fraction 4 (5.8 mg) contained two components which were correlated chromatographically with 2,3-di-, and 3,4-di-*O*-methyl-D-glucose. They were present in the ratio of 3:1.

Molar Ratio of Residues in US-3 Dextran.—2,3,4,6-Tetra-, 2,3,4-tri-, 2,4-di-, and 2,3-di- + 3,4-di-*O*-methyl-D-glucose were present in the molar ratio of 1:9.3:1.2:0.3 + a faint trace of an acidic-type component.

Periodate Oxidation of Clinical Dextrans

The procedure used was essentially that of Jeanes and Wilham (19). In the present work a series of determinations were carried out using sodium metaperiodate and another using the potassium salt. Oxidations at 5° with potassium metaperiodate were slow and generally either the sodium salt was used at 5° or the potassium salt at room temperature.

The formic acid was determined initially by titrating with sodium hydroxide using a pH meter potentiometrically but, as the results so obtained did not differ significantly from those obtained when methyl red was used as indicator, this latter method was adopted (13). The end points were stable. The periodate reduced was determined by the method of Fleury and Lange (9, 16).

In Table (II A and B) the values shown for "formic acid" refer to the number of moles of acid released per $C_6H_{10}O_5$ residue; those for "periodate reduced" refer to the number of moles reduced per $C_6H_{10}O_5$ residue.

TABLE II

(A) Clinical dextrans: oxidation by KIO_4 at room temperature (*ca.* 20°)

	Formic acid					Periodate reduced
Dextran	16	21	42	106	120	120 hours
C	—	0.78	—	0.90	0.90	1.90
E	0.43	0.84	0.88	0.89	0.89	1.91
S	—	0.39	0.66	0.83	0.84	1.90
US	—	0.83	0.87	0.87	0.88	1.95

(B) Fractions obtained by the ultrafiltration of a solution of E-dextran (see ref. 32): oxidation by $NaIO_4$ at 5° for 106 hours

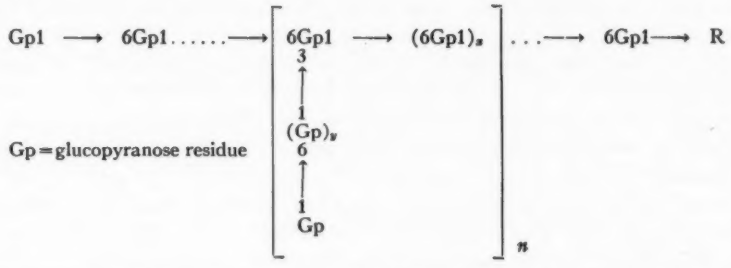
Fraction of			
$M_v \times 10^{-3}$*	$M_w \times 10^{-3}$†	Formic acid	Periodate reduced
213	344	0.88	1.93
133	225	0.87	1.95
102	171	0.87	1.98
39	—	0.90	2.04

*M_v = viscosity average molecular weight.
†M_w = weight average molecular weight.

The periodate-oxidized C, E, S, and US dextrans were dialyzed against running tap water and the non-dialyzable materials lyophilized and then hydrolyzed with *N* sulphuric acid (100°; 12 hours). The hydrolyzates were neutralized with barium carbonate, then filtered, and, after concentration, the filtrates were chromatographically examined. In all hydrolyzates a component was noted that was chromatographically indistinguishable from glucose. There was proportionately slightly more glucose in the hydrolyzate of the periodate-oxidized S-dextran than in those of the other periodate-oxidized dextrans.

Discussion

The studies on the methylated dextran fractions indicated that the clinical dextrans each contained a major fraction the molecules of which may be represented most simply by the following structure, where the symbol → denotes a glycosidic linkage between the hydroxyl groups on the carbon atoms indicated.



The average values of $x+y$, as derived from a study of those methylated fractions given in parenthesis, were 9 (C-3), 7.5 (E-2), 9 (S-2), 5 (S-3), and 9 (US-3). In each case the average value of x or y can lie between 0 and $x+y$. A small proportion of the molecules were probably terminated by a non-reducing "acidic-type" end group (R) (See Part 11). The ratio of non-reducing end-group residues to residues indicative of branching suggests that there was a marked degree of undermethylation in the S-3 fraction (as was known from the methoxyl determination), while the occurrence of mixtures of 2,3-di-, and 3,4-di-*O*-methyl-D-glucoses in the hydrolyzates of S-2 and US-3 indicated that they were also undermethylated but to a lesser degree, unless it be assumed that the molecules had branch points not only at C_3 but also at C_2 and at C_4. The hydrolyzates of C-3 and E-2 did not contain any 3,4-di-*O*-methyl-D-glucose and therefore the discovery of 2,3-di-*O*-methyl-D-glucose indicated that a few of the branch points (*ca.* 1 in 6–9) were at C_4 positions. Infrared studies (33, Part 11) on the clinical dextrans indicated that they contained α 1 → 6 and α 1 → 3 linkages.

The amounts of formic acid released upon periodate oxidation of the C, E, S, and US dextrans were compatible with the molecules having respectively 9, 8, 5, and 8 non-terminal glucopyranose residues for every non-reducing end group. This accords reasonably well with the values obtained

by studies on the methylated fractions C-3, E-2, S-3, and US-3. It should be noted, however, that the major methylated S-dextran fraction, i.e. S-2, gave a value of 9. The amounts of periodate reduced were in all cases much higher than could be accounted for by a structure possessing 1→3 rather than 1→4 branch points. It has already been noted that similarly anomalous results were remarked upon by Van Cleve *et al.* Assuming complete oxidation, the isolation in the present work of glucose from the hydrolyzates of the various periodate-oxidized dextrans indicated that the branch points were on C_3 positions. Periodate-oxidation studies on fractions obtained by the ultrafiltration of a solution of E-dextran (32) indicated that the molecules on the average had a ratio of 7 to 9 non-terminal glucopyranose residues to every non-reducing end group. The periodate reduced was again in excess of that to be expected for a molecule principally branched at C_3 positions. It should, however, be noted that the E-dextran used in methylation studies and that used in ultrafiltration studies were from different lot numbers.[4]

In work on clinical dextrans, in contrast to that on native polysaccharides, there may be an additional, complicating factor. It is possible that, during depolymerization of the native dextran, molecules of structurally different types may have been produced. If that were the case then the fractionation of the methylated dextran would depend not only on the degree of methylation and the molecular size of the various molecules, but also upon basic differences in molecular structures. May and Zakrewski (21) determined the electrophoretic distribution patterns of E-, S-, and US-type clinical dextrans in borate buffer and noted that all gave two peaks. An inference of structural heterogeneity was made earlier by Wilkie *et al.* (32) with respect to the E-dextran. While the periodate oxidation studies relate to clinical dextran, it is possible that the structural studies based on methylation should be related only to the structure of the parent molecules of the methylated material in each fraction studied. That there is a fairly wide difference in molecular structure is indicated by the difference in the ratios of terminal to non-terminal residues in the S-2 and S-3 methylated dextran fractions. This difference cannot be solely ascribed to the degree of undermethylation of the S-3 fraction, even if the improbable assumption should be made that the undermethylations were exclusively associated with the terminal residues.

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References

1. Barker, S. A., Bourne, E. J., Bruce, G. T., and Stacey, M. Chem. & Ind. (London), 1156 (1952).
2. Barker, S. A., Bourne, E. J., James, A. E., Brock Neely, W., and Stacey, M. J. Chem. Soc. 2096 (1955).

3. BARKER, S. A., BOURNE, E. J., BRUCE, G. T., BROCK NEELY, W., and STACEY, M. J. Chem. Soc. 2395 (1954).
4. BIXLER, G. H., HINES, G. E., MCGHEE, R. M., and SHURTER, R. A. Ind. Eng. Chem. **45**, 692 (1953).
5. BRAUNS, F. E. Can. J. Research, B, **16**, 73 (1938).
6. CHARLES, A. F. and FARRELL, L. N. Can. J. Microbiol. **3**, 239 (1957).
6*a*. CHRISTENSEN, E. M. and SMITH, F. J. Am. Chem. Soc. **79**, 4492 (1957).
7. CORBETT, W. M. and KENNER, J. J. Chem. Soc. 3281 (1954).
8. EASTERBROOK, W. C. and HAMILTON, J. B. Analyst, **78**, 551 (1953).
9. FLEURY, P. and LANGE, J. J. pharm. chim. **17**, 107 (1933).
10. FOWLER, F. L., BUCKLAND, I. K., BRAUNS, F., and HIBBERT, H. Can. J. Research, B, **15**, 486 (1937).
11. FREUDENBERG, K. and BOPPEL, H. Ber. **71**, 2505 (1938).
11*a*. GEERDES, J. D., LEWIS, B. A., and SMITH, F. J. Am. Chem. Soc. **79**, 4209 (1957).
12. GRÖNWALL, A. Dextran and its use in colloidal infusion solutions. Almqvist & Wiksell, Stockholm. 1957.
13. HALSALL, T. G., HIRST, E. L., and JONES, J. K. N. J. Chem. Soc. 1399 (1947).
14. HOUGH, L., JONES, J. K. N., and WADMAN, W. H. J. Chem. Soc. 1702 (1950).
15. INGELMAN, B. Uppsala Läkareförenings Förhandlingar, **54**, 107 (1949).
16. JACKSON, E. L. Organic reactions. Vol. 2. *Edited by* R. Adams. John Wiley and Sons, Inc., New York. 1944. p. 341.
17. JEANES, A. Dextran, a selected bibliography. U.S. Dept. of Agr. AIC-288 (Revised). 1952.
18. JEANES, A., HAYNES, W. C., WILHAM, C. A., RANKIN, J. C., MELVIN, E. H., AUSTIN, M. J., CLUSKEY, J. E., FISHER, B. E., TSUCHIYA, H. M., and RIST, C. E. J. Am. Chem. Soc. **76**, 5041 (1954).
19. JEANES, A. and WILHAM, C. A. J. Am. Chem. Soc. **72**, 2655 (1950).
20. MACHELL, C. and RICHARDS, G. N. J. Chem. Soc. 1199 (1958).
21. MAY, Z. and ZAKRZEWSKI, K. Experientia, **14**, 35 (1958).
22. MUSKAT, I. E. J. Am. Chem. Soc. **56**, 693 (1934).
23. PARTRIDGE, S. M. and WESTALL, R. G. Biochem. J. **42**, 238 (1948).
24. RANKIN, J. C. and JEANES, A. J. Am. Chem. Soc. **76**, 4435 (1954).
25. SLOAN, J. W., ALEXANDER, B. H., LOHMAR, R. L., WOLFF, I. A., and RIST, C. E. J. Am. Chem. Soc. **76**, 4429 (1954).
26. SQUIRE, J. R., BULL, J. P., MAYCOCK, W. D'A., and RICKETTS, C. R. Dextran, its properties and use in medicine. Blackwell Scientific Publications, Ltd., Oxford. 1955.
27. STACEY, M. and SWIFT, G. J. Chem. Soc. 1555 (1948).
28. VAN CLEVE, J. W. and SCHAEFER, W. C. J. Am. Chem. Soc. **77**, 5341 (1955).
29. VAN CLEVE, J. W., SCHAEFER, W. C., and RIST, C. E. J. Am. Chem. Soc. **78**, 4435 (1956).
30. VIEBOCK, F. and BRECKER, G. Ber. **63**, 3207 (1930).
31. WEISSBERG, S. G. Intercomparison of British and American commercial dextrans, U.S. Department of Commerce, National Bureau of Standards Report No. 3997, Washington, D.C. 1955.
32. WILKIE, K. C. B., JONES, J. K. N., EXCELL, B. J., and SEMPLE, R. E. Can. J. Chem. **35**, 795 (1957).
33. WILKIE, K. C. B. and JONES, J. K. N. To be published.
34. WOLFF, I. A., MEHLTRETTER, C. L., MELLIES, R. L., WATSON, P. R., HOFREITER, B. T., PATRICK, P. L., and RIST, C. E. Ind. Eng. Chem. **46**, 370 (1954).
35. Brit. Patent No. 719, 382 (Example 1).

Appendix

Manufacture of Clinical Dextrans

The C-dextran and the E-dextran were prepared industrially by the methods reported by Charles *et al.* (6) and Pautard and Stacey (35) respectively, while the S-dextran was prepared by the method of Ingelman (15) and the US-dextran by a similar method (4, 34). The partial depolymerization of the native dextrans was effected in the case of the C-dextran by enzymatic hydrolysis, in that of the E-dextran by thermal degradation, and in those of the S-dextran and the US-dextran by acidic hydrolysis.

Specifications Supplied by the Manufacturers

C-Dextran (results supplied by Dr. A. F. Charles):

Relative viscosity 4.045
Intrinsic viscosity 0.27

E-Dextran (results supplied by Dr. A. R. Lockwood): From the viscosity data, in conjunction with results obtained from other fractions by light scattering measurements, it was inferred that Lot No. 54/091 had M_w:

Mean	135,000
Upper 10%	300,000
Lower 10% less than	30,000

(See also values determined by ultrafiltration studies and comments in Part 11).

S-Dextran (results determined by Dr. K. Granath on a clinical dextran prepared in a similar way to Batch B-214):

%	η_{20}	M_w
9.15	0.358	168,000
11.29	0.352	160,000
10.98	0.170	28,700
10.0	0.167	27,400
Average η_{25}	0.260	
Average M_w	82,000	

US-Dextran (results supplied by Dr. Homer E. Stavely):

Intrinsic viscosity	0.2192	
Viscosity	3.42 centipoises at 25° C	
Mol. wt. by light scattering:	Whole polymer	77,000
	High fraction (10.01%)	168,400
	Low fraction (9.98%)	28,400

ESTIMATION OF CORTICOSTEROIDS BY THE REDUCTION OF FERRICYANIDE[1]

N. R. STEPHENSON

Abstract

A procedure based on a modification of Folin's micromethod for blood sugar (1, 2) was used to investigate the reducing activity of various corticosteroids. The ferrocyanide produced as a result of the reduction of ferricyanide in alkaline solution was measured photometrically as Prussian blue. With a filter transmitting light at 620 mμ, the relation between the absorbance of the chromogen and the amount of the reducing steroid obeyed Beer's law over the range from 0.005 to 0.050 mg. The oxygen function at C-3 accounted for most of the reducing power of the non-alpha ketolic steroids studied. An oxygen function at C-11 did not affect significantly the reduction of ferricyanide by 17-desoxycorticosteroids. Although the presence of a hydroxyl at C-17 depressed the reducing activity of the alpha-ketol side chain, a fluorine at C-9 and an hydroxyl at C-11 appeared to overcome this inhibiting effect. Evidence was obtained to suggest that a C-16 hydroxyl group was able to increase the reducing action of the alpha-ketolic side chain. Esterification of the C-21 hydroxyl influenced the reduction of ferricyanide only when interference with hydrolysis of the ester in the alkaline medium was experienced.

Introduction

Early investigators of adrenal corticosteroids revealed that biological activity was closely associated with the presence of a primary alpha-ketol grouping which imparted strongly reducing properties to the molecule (3, 4). This characteristic has been employed as the basis of several procedures for the determination of corticosteroids in biological fluids. These include the reduction of copper (5), phosphomolybdic acid (6), ferricyanide (7), and tetrazolium salts (8).

The method to be described in this paper is based on a modification of Folin's micromethod for blood sugar (1, 2). The alpha-ketolic side chain of the adrenal corticosteroid is oxidized with alkaline potassium ferricyanide, and the ferrocyanide thus produced is measured photometrically as Prussian blue. A linear relationship has been established between the absorbance of the chromogen and the amount of the reducing steroid over the range from 0.005 to 0.050 mg.

Experimental

Reagents

All reagents were prepared from analytical grade chemicals and water distilled from an all-glass apparatus. The glassware employed in the determinations was cleaned with Haemo-Sol.[2]

(*a*) *Potassium ferricyanide solution.*—0.1% $K_3Fe(CN)_6$ in water.

(*b*) *Sodium carbonate solution.*—0.23% anhydrous Na_2CO_3 in water.

(*c*) *Ferric iron solution.*—Dissolve 2.0 g $FeNH_4(SO_4)_2.12H_2O$ in 945 ml of water and add 55 ml of 85% H_3PO_4 (9).

[1]Manuscript received January 22, 1958.
Contribution from the Laboratory of the Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Canada.

[2]Haemo-Sol is distributed by Meinecke and Company Inc., New York.

(*d*) *95% ethyl alcohol (aldehyde-free).*—(i) to 1000 ml of redistilled 95% ethyl alcohol add 4 g of *m*-phenylenediamine dihydrochloride and allow to stand for 1 week in the dark with occasional mixing. Distil through a Vigreux column rejecting the head and tail fractions and collecting only the middle portion (Rowe and Phelps (10); Callow *et al.* (11)). (ii) To 1000 ml of redistilled 95% ethyl alcohol, add 2 g 2,4-dinitrophenylhydrazine and 1.3 ml concentrated hydrochloric acid (sp. gr. 1.19); allow to stand in the dark with occasional shaking for at least 48 hours. Distill through a Vigreux column discarding the first and the last 100 ml of the distillate. Repeat the distillation procedure, collecting only the middle portion of the distillate (Sobel *et al.* (12)). Treatment of 95% ethyl alcohol by either method (i) or (ii) was found to provide a product which remained aldehyde-free for at least 2 weeks when kept in the dark at refrigerator temperature.

(*e*) *Stock solutions of the steroids.*—Dissolve an accurately weighed amount of the steroid in sufficient 95% ethyl alcohol (aldehyde-free) to yield 1.00 mg per ml.

(*f*) *Dilute solutions of the steroids.*—Dilute the stock solution of the steroid with 95% ethyl alcohol (aldehyde-free) to provide solutions containing from 0.010 to 0.100 mg per ml.

Method

Add 0.5 ml of the dilute solution of the steroid to a 10-ml glass-stoppered graduated cylinder containing 0.5 ml of the 0.1% potassium ferricyanide solution. Add 1.0 ml of the 0.23% solution of sodium carbonate and mix carefully. Immerse the cylinders to a depth of approximately 4 cm in a 60° C constant temperature water bath. During the heating period, stopper the cylinders to prevent evaporation and reduce oxidation of the reaction mixture. After heating the contents for exactly 60 minutes, cool them by immersing the cylinders in cold tap water (approximately 15° C) for 1 to 2 minutes. Add 1.0 ml of the ferric iron solution and mix by gentle swirling. Allow the Prussian blue color to develop for 15 minutes, then dilute the reaction mixture with water to either 6 or 10 ml depending upon the amount of steroid.[3] Measure the absorbance by means of a suitable photoelectric colorimeter equipped with a filter transmitting light at 620 mμ.[4] Prepare a reagent blank at the same time as the samples under test using 0.5 ml of the 95% ethyl alcohol (aldehyde-free).

Results and Discussion

Effect of Temperature on the Rate of Reduction of Alkaline Ferricyanide by Corticosteroids

In some of the procedures described (5, 6, 7) for measuring reducing steroids, the reaction was carried out in a boiling water bath at 100° C for periods of

[3]A total volume of 6 ml was used for quantities of steroid below 0.02 mg. The 10-ml volume was employed when the amount of the steroid ranged from 0.01 to 0.05 mg.

[4]The Evelyn photoelectric colorimeter (Rubicon Co., Philadelphia) equipped with Filter No. 620 (transmission limits 595 to 660 mμ) was used in this work. The code number (620) of the filter represents the mean wavelength in mμ of the transmitted band.

time ranging from 3 minutes to 1 hour. In the present study when the rate of reduction of the alkaline ferricyanide by a corticosteroid was determined at 100° C, the ethyl alcohol in the reaction mixture invariably boiled off during the heating period. In order to avoid this ebullition of the solvent, the rate of reduction of the ferricyanide was investigated at 75° C and at 60° C. A water-bath temperature of 75° C permitted the use of ethyl alcohol as the steroid solvent, while 60° C was suitable for reaction mixtures containing either methyl alcohol or ethyl alcohol. Figure 1 shows the rate of increase of the Prussian blue color produced as a result of the reduction of alkaline ferricyanide by 0.5 ml of a 0.2 millimolar (m*M*) solution of desoxycorticosterone acetate at 60° C, 75° C, and 100° C for periods of time ranging from 5 to 120 minutes. Although color formation was most rapid at 100° C, it was not complete after 30 minutes at this temperature. On the other hand when the rate of reduction was measured at 60° C or 75° C, the absorbance tended to approach a maximum value as the time in the water bath was increased. At 60° C for example, the rate of change in the absorbance was very slow when the time of incubation was extended beyond 60 minutes. In practice, the absorbance values were found to be reproducible when the heating period was measured accurately for 60 minutes at 60° C or 30 minutes at 75° C.

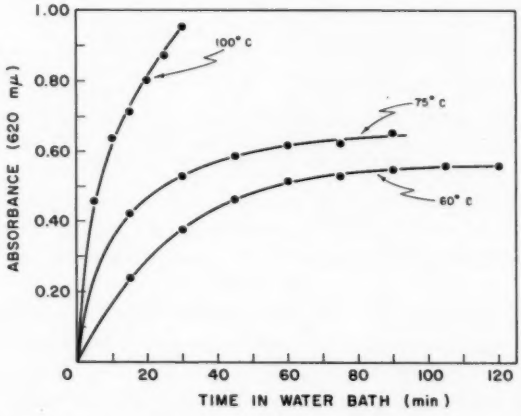
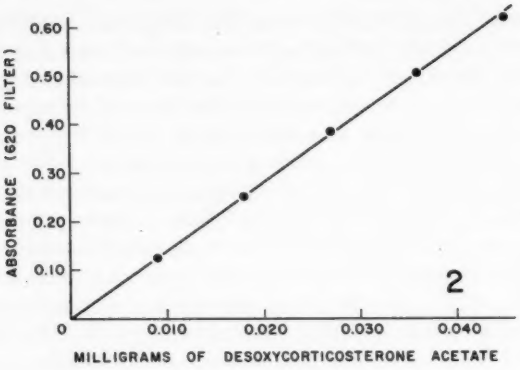
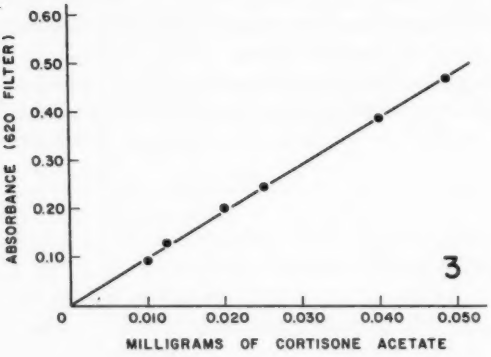


FIG. 1. Rate of reduction of alkaline ferricyanide by 0.5 ml of a 0.2 m*M* solution of desoxycorticosterone acetate at 60° C, 75° C, and 100° C.

Relationship between the Absorbance of the Chromogen and the Amount of the Corticosteroid

The results obtained by plotting the absorbance of the Prussian blue chromogen against the weight of desoxycorticosterone acetate are shown in Fig. 2. The heating time was 60 minutes at 60° C and the final volume of the reaction mixture was 10 ml. Figures 3 and 4 illustrate the relationship found with the 17-hydroxycorticosteroids: cortisone acetate and hydrocortisone. In these experiments, the heating time was 30 minutes at 75° C.





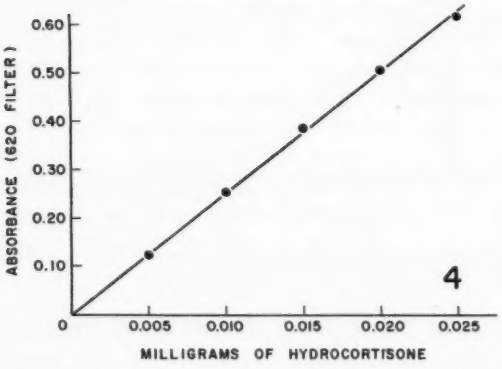


FIG. 2. Relationship between the absorbance and the amount of desoxycorticosterone acetate. The heating period was 60 minutes at 60° C. The final volume of the reaction mixture was 10 ml.

FIG. 3. Relationship between the absorbance and the amount of cortisone acetate. The heating period was 30 minutes at 75° C. The final volume of the reaction mixture was 10 ml.

FIG. 4. Relationship between the absorbance and the amount of hydrocortisone. The heating period was 30 minutes at 75° C. The final volume of the reaction mixture was 6 ml.

TABLE I

Beer's law constant (K) for hydrocortisone and corticosterone (volume = 10 ml)

Weight of steroid, C (mg)	Hydrocortisone, K	Corticosterone, K
0.010	11.1	12.2
	11.6	11.9
0.020	12.8	13.6
	12.7	14.2
0.040	12.0	14.4
	12.0	14.4
0.050	12.0	—
Average	12.0±0.6*	13.4±1.1*

*Standard deviation (s).

TABLE II

Beer's law constant (K) for the acetate esters of cortisone, hydrocortisone, and desoxycorticosterone (volume = 6 ml)

Weight of steroid, C (mg)	Cortisone acetate, K	Hydrocortisone acetate, K	Desoxycorticosterone acetate, K
0.005	18.6	20.0	22.4
0.010	19.6	22.0	26.8
0.015	20.1	22.3	24.9
0.020	18.4	21.0	24.9
0.025	18.1	20.9	24.8
Average	19.0±0.8*	21.2±0.9*	24.8±1.6*

*Standard deviation (s).

With cortisone acetate (Fig. 3) the final volume was 10 ml, while with hydrocortisone (Fig. 4), the reaction mixture was diluted to 6 ml. Figures 2, 3, and 4 show that the relationship between the absorbance of the chromogen and the weight of the corticosteroid is linear under the experimental conditions outlined in the method. The applicability of Beer's law to this relationship may be demonstrated by calculating the constant K, derived from the equation:

$$KC = A$$

where C is the weight of the steroid in mg and A is the absorbance[5] obtained with Filter No. 620. Table I indicates that the values for the Beer's law constant, K, estimated from data obtained for hydrocortisone and corticosterone are relatively constant. In this experiment the final volume of the reaction mixture was 10 ml and the heating period was 30 minutes at 75° C. Table II illustrates the values for the constant K obtained for the acetate esters of cortisone, hydrocortisone, and desoxycorticosterone when the volume of the reaction mixture was reduced to 6 ml. The standard deviations shown with the arithmetic means in Tables I and II provide an estimate of the variability of the values for K calculated for various amounts of the corticosteroids. A coefficient of variation[6] of ± 4.9 was estimated from a series of 10 replicate determinations of the K value for desoxycorticosterone acetate.

[5]Absorbance = 2 − log of the galvanometer reading.

[6]Coefficient of variation = $\frac{\text{standard deviation } (s) \times 100}{\text{arithmetic mean } (\bar{x})}$.

Relative Reducing Activity of the Corticosteroids

In addition to showing adherence to Beer's law, the values for K described in Tables I and II indicate clearly that not all steroids with an alpha-ketol group reduce alkaline ferricyanide to the same extent under comparable experimental conditions. The reducing activity of various steroids on an equimolar basis is given in Table III. The absorbance of the Prussian blue chromogen was employed as the criterion of the reducing power of the steroid. Corticosterone and 11-dehydrocorticosterone had values similar to that of 11-desoxycorticosterone suggesting that the oxygen-function at C-11 did not influence the ability of the alpha-ketol side chain to reduce alkaline ferricyanide. On the other hand, the presence of a C-17 hydroxyl group appeared to depress the reducing action of the alpha-ketolic grouping because 17-desoxycorticosteroids were, on the average, more strongly reducing than 17-hydroxycorticosteroids. Heard and Sobel (6), using the phosphomolybdic acid reagent, made a similar observation; Mader and Buck (8) working with tetrazolium salts in an alkaline medium also mentioned this effect of the hydroxyl group in the 17-position. Tables II, III, and IV show that 17-hydroxycorticosteroids with a C-11 hydroxyl group were able to reduce ferricyanide to a greater extent than those with a ketone group at C-11. In addition, according to Table III, the reducing power of 9 alpha-fluorohydrocortisone approached that of the 17-desoxycorticosteroids. These observations suggest that both the C-9 fluorine and the C-11 hydroxyl were able to bring about this apparent increase in activity by counteracting at least in part the depressing effect of the hydroxyl group at C-17.

TABLE III

Absorbance of the chromogen produced by 0.5 ml of a 0.2 mM solution of the steroid

Steroid	Absorbance at 620 mμ	
	60° C for 60 min	75° C for 30 min
Corticosterone	0.52	0.54
Dehydrocorticosterone	0.52	0.54
Desoxycorticosterone	0.52	0.53
Dihydrodesoxycorticosterone	0.49	0.50
Hydrocortisone	0.44	0.47
Cortisone	0.40	0.41
Desoxycortisone	0.42	0.41
Tetrahydrocortisone	0.35	0.34
Prednisolone	0.44	0.49
Prednisone	0.40	0.40
9α-Fluorohydrocortisone	0.50	0.54
16α-Hydroxy-9α-fluoro-prednisolone (triamcinolone)	0.70	0.76
Progesterone	0.01	
Methyltestosterone	0.02	
Testosterone propionate	0.02	
Adrenosterone	0.04	
Pregnenolone acetate	0.006	
Estrone	0.25	

The fact that tetrahydrocortisone and dihydrodesoxycorticosterone displayed lower absorbance values than cortisone and desoxycorticosterone respectively indicated that the conversion of the ketone at C-3 to a hydroxyl group decreased the reducing activity of the corticosteroid. Examination of the data in Table III suggested that the C-3 ketone group could account for approximately 5 to 10% of the total reducing power of the corticosteroids. This conclusion was supported by the observation that pregnenolone acetate, a steroid that has neither an alpha-ketol side chain nor a ketone group in the 3-position had the lowest reducing activity of any of the steroids tested during the course of this study.

The double bond between C-1 and C-2 did not appear to have any influence on the reduction of ferricyanide because the 1-dehydro derivatives, prednisone and prednisolone, had approximately the same absorbance values as cortisone and hydrocortisone respectively. Triamcinolone, a derivative of prednisolone possessing a 9 alpha-fluorine and a 16 alpha-hydroxyl group, was found to be the most active reducing steroid of all those tested in this series. The data suggested that the presence of the hydroxyl group at C-16 adjacent to the alpha-ketol side chain was probably responsible for this marked increase in reducing activity.

The steroids lacking an alpha-ketol side chain at C-17, with the exception of estrone, reduced the alkaline ferricyanide to a very limited extent. The presence of the Δ^4-3-keto grouping can probably account for all of the reducing activity obtained with these compounds. Estrone and possibly other phenolic estrogens, which might interfere with the estimation of corticosteroids, can be

TABLE IV

Effect of esterification at C-21 on the absorbance of the Prussian blue color produced by 0.5 ml of a 0.2 m*M* solution of the steroid

Steroid	Absorbance at 620 mμ	
	60° C for 60 min	75° C for 30 min
Desoxycorticosterone	0.52	0.53
Desoxycorticosterone acetate	0.52	0.53
Dihydrodesoxycorticosterone	0.49	0.50
Dihydrodesoxycorticosterone hemisuccinic acid	0.47	0.49
Cortisone	0.40	0.41
Cortisone acetate	0.38	0.41
Hydrocortisone	0.44	0.47
Hydrocortisone acetate	0.44	0.47
Hydrocortisone *t*-butylacetate	0.35	0.40
Prednisolone	0.44	0.49
Prednisolone acetate	0.45	0.51
Prednisolone hemisuccinic acid	0.44	0.50
21-Piperidino-prednisolone	0.44	0.50
Prednisolone diethylaminoacetate	0.49	0.54
Prednisolone-21-phosphate	0.05	0.08
Triamcinolone	0.70	0.76
Triamcinolone diacetate	0.65	0.70

removed from a chloroform extract by washing with a 2 *N* sodium hydroxide solution. Table III also demonstrates that the reducing power of the steroids when heated for 30 minutes at 75° C was only slightly higher than that observed when the heating period was 60 minutes at 60° C.

Table IV compares the reducing activity of esterified corticosteroids with that of the free alcohols. The acetate esters of desoxycorticosterone, cortisone, hydrocortisone, and prednisolone had essentially the same effect on the alkaline ferricyanide as the free compounds. However, esterification of triamcinolone with acetate at both C-16 and C-17 resulted in a small decrease in the reducing activity. The *t*-butyl acetate ester of hydrocortisone was somewhat less active than either the acetate or the alcohol suggesting that this compound was not completely hydrolyzed during the heating period. Although the ability of the hemisuccinic acid ester of dihydrodesoxycorticosterone to reduce ferricyanide was slightly less than that of the free alcohol, no such effect was observed with the prednisolone hemisuccinate ester. While esterification of prednisolone with diethylaminoacetate at C-21 appeared to increase the reducing power to some extent, the introduction of a piperidyl group at the 21-position had no effect on the reducing activity. In contrast, the absorbance obtained for prednisolone-21-phosphate was of the same order as that experienced with steroids lacking an alpha-ketol side chain at C-17. It is concluded from this finding that the C-21 phosphate ester was not hydrolyzed by the alkaline reaction mixture employed for the determination.

Acknowledgments

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References

1. FOLIN, O. J. Biol. Chem. **77**, 421 (1928).
2. FOLIN, O. and MALMROS, H. J. Biol. Chem. **83**, 115 (1929).
3. REICHSTEIN, T. and SHOPPEE, C. W. Vitamins and hormones. Vol. 1. Academic Press Inc., New York. 1943. p. 345.
4. MASON, H. L., MYERS, C. S., and Kendall, E. C. J. Biol. Chem. **116**, 267 (1936).
5. TALBOT, N. B., SALTZMAN, A. H., WIXON, R. L., and WOLFE, J. K. J. Biol. Chem. **160**, 535 (1945).
6. HEARD, R. D. H. and SOBEL, H. J. Biol. Chem. **165**, 687 (1946).
7. GORDON, J. J. and PELLY, J. E. Nature, **168**, 603 (1951).
8. MADER, W. J. and BUCK, R. R. Anal. Chem. **24**, 666 (1952).
9. KINGSLEY, G. R. and REINHOLD, J. G. J. Lab. Clin. Med. **34**, 713 (1949).
10. ROWE, A. W. and PHELPS, E. P. J. Am. Chem. Soc. **46**, 2078 (1924).
11. CALLOW, N. H., CALLOW, R. K., EMMENS, C. W., and STROUD, S. W. J. Endocrinol. **1**, 76 (1939).
12. SOBEL, C., GOLUB, O. J., HENRY, R. J., JACOBS, S. L., and BASU, G. K. J. Clin. Endocrinol. and Metabolism, **18**, 208 (1958).

STUDIES ON THE TIME COURSE OF THE RESPONSE OF THE ADRENAL CORTEX TO HISTAMINE AND COLD[1]

E. SCHÖNBAUM, W. G. BRUCE CASSELMAN, AND ROSEMARY E. LARGE

Abstract

Rats were injected with histamine or exposed to cold. In either case there was a transient elevation in the output of corticosteroids by the excised adrenal glands in vitro but a prolonged fall in adrenal ascorbic acid. The return of steroid formation to pre-exposure or even lower rates within three hours was not accompanied by any further change of the concentration of adrenal ascorbic acid.

Introduction

Previously, it was reported that the administration of histamine to rats causes certain changes in their adrenal glands: a decrease in the concentration of ascorbic acid, an increase in the rate of steroid formation in vitro, and cytological evidence of adrenocortical stimulation (1). In that investigation, the observations were made at only one time, 30 minutes, after the intraperitoneal injection of histamine. Further studies, at various times up to 3 hours, have now been completed giving some indication of the kinetics of the adrenocortical responses not only to histamine but also to exposure to cold. In addition, the effects of nembutal and morphine upon steroid formation in response to histamine have been studied since the consecutive administration of these drugs prevents the decrease in adrenal ascorbic acid after injury (2, 3).

Methods

Male rats of the Wistar strain (Woodlyn Farms, Guelph, Ontario) were used. Histamine dihydrochloride (1% in 0.85% NaCl) was given intraperitoneally, in a dose of 10 mg per 100 g body weight. Control rats were similarly injected with saline. When used, nembutal (4 mg/100 g) and morphine (2 or 4 mg/100 g) were given intraperitoneally, 40 and 30 minutes respectively before the histamine. Rats that were exposed to cold were placed in a deep-freeze at $-20°$ C for as long as 3 hours. In all experiments the rats were killed by decapitation. Their adrenals were removed as quickly as possible and immediately cleaned, weighed, and prepared for the chemical or cytological investigations.

The methods for assessing the rate of formation of corticosteroids and the concentration of adrenal ascorbic acid, and the cytological procedures, have been described previously (1). The rate of formation of corticosteroids in vitro is expressed as the equivalent micrograms of hydrocortisone released into the medium per 100 mg of fresh adrenal tissue per hour.

[1]Manuscript received August 7, 1958.
Contribution from the Banting and Best Department of Medical Research and the Department of Anatomy, University of Toronto, Toronto, Canada.

Results

The effects of histamine were studied in groups of 10 rats that were decapitated immediately or 15, 30, 60, or 180 minutes after injection. The results are presented in Table I. In the rats killed 15 to 60 minutes after injection, the rates of steroid production in vitro were significantly greater than in the rats killed immediately. In the rats killed 3 hours after injection, however, the rate was significantly less than in those killed immediately. The adrenal ascorbic acid concentrations decreased throughout the experiment. The glands from the rats given histamine were not studied cytologically.

The results in Table II show that, in the doses given, nembutal plus morphine do not prevent stimulation of adrenocortical activity by histamine whether ascorbic acid concentration (2, 3) or the rate of steroid formation

TABLE I

Effect of histamine on adrenal activity

Minutes after injection	Steroid formation, μg/100 mg/hour	Ascorbic acid concentration, μg/100 mg	r*	P*
0	13±1.1†	375±11.4	–	–
15	22±2.0	305±13.4	−0.698	0.01
30	24±1.9	277± 9.9	−0.674	0.01
60	19±1.8	233±11.6	−0.539	0.05
180	8±0.4	198± 6.3	+0.664	0.01

*r and P values refer to the significance of the correlation between changes in steroid formation and ascorbic acid contents; 10 rats per group.
†Mean ± S.E.

TABLE II

Effects of nembutal, morphine, and histamine on adrenal activity

	Time in minutes					Steroid formation/hr	Ascorbic acid concentration
	0	10	40	70	n	μg per 100 mg adrenal tissue	
A.	N	S	—	D	10	11±1.2	397±11
	N	M	—	D	10	13±0.8	379±28
	N	S	H	D	9	23±1.6	331±25
	N	M	H	D	13	22±2.0	302±15
B.	N	H	D	—	6	30±2.4	293±22
	N	S	—	D	6	10±4.9	366±28
C.	S	S	—	D	5	7±0.8	314±31
	S	MM	—	D	5	17±2.9	216± 6
	N	MM	—	D	5	8±1.6	418±22

NOTE: Abbreviations:
N—nembutal, 4 mg/100 g body wt.
S—saline.
M—morphine sulphate, 2 mg/100 g body wt.
MM—morphine sulphate, 4 mg/100 g body wt.
H—histamine dihydrochloride, 10 mg/100 g body wt.
D—decapitation.

in vitro is taken as the index. Nembutal alone does not stimulate the formation of steroids under the conditions of these experiments; it prevents the increased steroid formation caused by morphine.

In rats exposed to −20° C there is a significant increase in the rate of steroid formation only in those exposed for 30 minutes. As is shown in Table III, the steroid production does not differ significantly from control values in the rats exposed for 60 or 180 minutes. However, adrenal glands, already stimulated by exposure of the rats to cold for these periods, are still able to respond with a further increase of the rate of formation of steroids when corticotrophin is added to the incubation medium. Table III also shows that, after exposure to cold as well as after histamine injections, the ascorbic acid concentration falls during the first hour and then remains low.

Cytological assessment of adrenocortical stimulation was based on the changes in the lipid droplets within the individual cells. Following stimulation, the previously moderately large, discrete droplets are more numerous and smaller, even apparently submicroscopic in size (4). Such changes were seen in the cells in the *Zona fasciculata* and *Zona reticularis* particularly in the rats exposed to −20° C for 30 minutes. In many of the rats exposed for a longer time, only slight or no changes were noted.

Adrenocortical activity was also studied in rats exposed to less severe cold (0° to +2° C) for 1, 3, 17, or 28 days. At these times the rates of formation of steroids were found to be 14, 16, 19, and 21 μg/100 mg adrenal tissue/hour, while corresponding control (room temperature) rates were 13, 15, 14, and 13 μg.

The increased corticosteroid production in vitro, the ascorbic acid depletion, and the cytological evidence of the activation noted in the rats killed 30 minutes after the administration of histamine (10 mg/100 g body weight) are similar to the changes already reported for the same time after a larger dose (50 mg/100 g body weight) of the drug (1). It is customary to regard either ascorbic acid depletion or increased steroid production as evidence of adrenocortical stimulation. However, the data in Table I show that changes in steroid production and ascorbic acid concentration do not necessarily occur at the same time or at the same rate. Thus, following the administration of histamine the rate of steroid formation in vitro passes through a maximum at 30 minutes whereas the ascorbic acid concentration falls during the first hour and then remains low for at least two more hours. It might be suggested that the rate of steroid formation in vitro reflects adrenocortical activity at the time the gland was removed whereas the ascorbic acid concentration merely provides evidence of adrenocortical stimulation (5), that is, of increased blood levels of corticotrophin (6), at some time previously. The relation of the prompt change in ascorbic acid to the biogenesis of corticosteroids is not known (7).

The observations made 3 hours after the injection of histamine show that at that time the pronounced ascorbic acid depletion was not associated with any increased rate of steroid formation in vitro. Despite the limitations of

TABLE III

The effect of acute cold exposure on adrenocortical activity

Treatment	No. animals	Steroid output in vitro/hr				Ascorbic acid	
		−ACTH	*t.P**	+ACTH†	*t.P*	Concn.	*t.P*
Room temperature	40	12.8‡ ±0.9	— —	23.2‡ ±1.6	— —	318‡ ±10	— —
−20° C; 30 min	12	19.3 ±1.2	2.75 0.01	23.7 ±1.5	n.s.	255 ±13	2.42 0.02
−20° C; 60 min	9	11.8 ±1.7	n.s.	28.6 ±3.4	n.s.	204 ±13	4.23 0.001
−20° C; 180 min	12	11.2 ±1.5	n.s.	18.1 ±2.8	n.s.	212 ±12	4.24 0.001

**t* and *P* values refer to the significance of the difference between cold-exposed and control rats.
†Corticotrophin (Connaught) = 0.2 I.U. per 100 mg adrenal tissue.
‡Mean ± S.E., μg per 100 mg adrenal tissue.

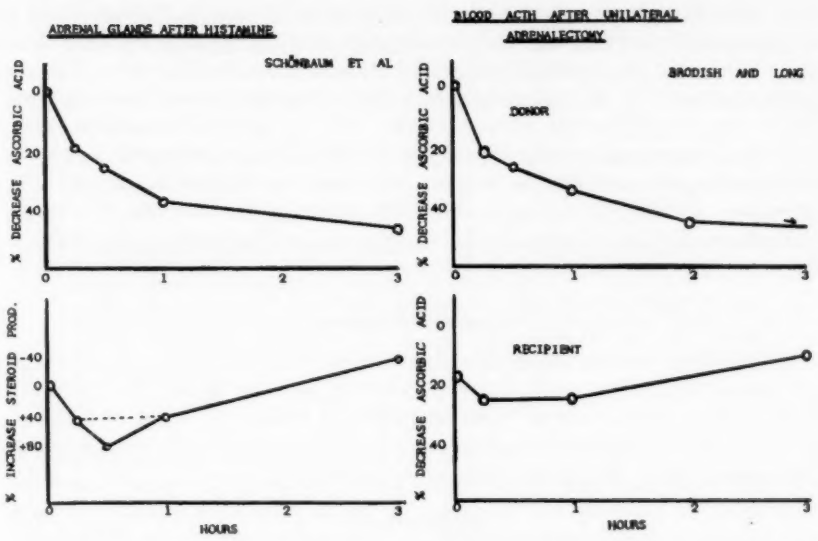

FIG. 1. A comparison of the time courses of adrenal ascorbic acid depletion and steroid formation in vitro in histamine-treated rats with that of adrenal ascorbic acid depletion and blood corticotrophin in unilaterally adrenalectomized rats (6). Values were taken from Table I of this paper and from Table II, Brodish, A. and Long, C. N. H. Endocrinology, **59**, 666 (1956). These authors assayed blood corticotrophin by measuring the adrenal ascorbic acid depletion in hypophysectomized (recipient) rats after infusing blood from adrenalectomized (donor) rats (6). Note the parallelism between rates of steroid formation in vitro and levels of blood corticotrophin.

in vitro studies, such as the much lower rate of steroid production, it might be suggested that the rate of steroid production in vitro is an indication of the activity of adrenal tissue *in situ* and that the effect of endogenous corticotrophin persists in the excised tissue. Both these suggestions are supported by the observations on adrenal ascorbic acid and blood corticotrophin levels after unilateral adrenalectomy reported by Brodish and Long (6). In their rats, the adrenal ascorbic acid levels did not correspond with the concentrations of blood corticotrophin. This is shown in Fig. 1. This figure illustrates the striking agreement between the time course of the changes in the blood levels of corticotrophin in their animals on the one hand and the time course of the changes in the rate of in vitro steroid formation after histamine (Table I) on the other. While it is evident from both studies that the concentration of adrenal ascorbic acid in intact rats is not necessarily a suitable index of adrenocortical activity, this does not in any way detract from the immense value and the importance of the "Sayers assay" for corticotrophin using the hypophysectomized rat (8).

The transient increase in corticosteroid output noted in rats exposed to $-20°$ C rather resembles the early increase in the steroid concentration in the adrenal venous blood of unanesthetized dogs exposed to severe cold (9):

both returning to pre-exposure levels within 1 to 3 hours. Perhaps there is only an initial brief release of corticotrophin during exposure to cold for a short time. In the adrenal cortex the trophic hormone not only causes a sudden increase in steroid output but also activates adrenal carbohydrate (10, 11, 12, 13), lipid (14), phosphate (15, 16), and sulphur metabolism (17), as well as functional growth (18). These effects of corticotrophin might be more prolonged and might be of great importance in adaptation to cold (19). Moreover, prolonged exposure to 0–2° C causes a gradual rise of adrenocortical activity as indicated by the criteria used in this study. The relationship between acute and prolonged exposure to cold awaits further elucidation.

Acknowledgments

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References

1. SCHÖNBAUM, E. and CASSELMAN, W. G. B. Can. J. Biochem. and Physiol. **36**, 571 (1958).
2. BRIGGS, F. N. and MUNSON, P. L. Endocrinology, **57**, 205 (1955).
3. OHLER, E. A. and SEVY, R. W. Endocrinology, **59**, 347 (1956).
4. DEANE, H. W., SHAW, J. H., and GREEP, R. O. Endocrinology, **43**, 133 (1948).
5. LONG, C. N. H. Recent Progr. in Hormone Research, **1**, 99 (1947).
6. BRODISH, A. and LONG, C. N. H. Endocrinology, **59**, 666 (1956).
7. PINCUS, G. Bull. N.Y. Acad. Med. **33**, 587 (1957).
8. SAYERS, M. A., SAYERS, G., and WOODBURY, L. A. Endocrinology, **42**, 379 (1948).
9. EGDAHL, R. H. and RICHARDS, J. B. Am. J. Physiol. **185**, 239 (1956).
10. SCHÖNBAUM, E., BIRMINGHAM, M. K., and SAFFRAN, M. Can. J. Biochem. and Physiol. **34**, 527 (1956).
11. NOBLE, N. L. and PAPAGEORGE, E. Endocrinology, **57**, 492 (1955).
12. HAYNES, R. C., JR. and BERTHET, L. J. Biol. Chem. **225**, 115 (1957).
13. GLOCK, G. E. and MCLEAN, P. Biochem. J. **56**, 171 (1954).
14. HOLZBAUER, M. and VOGT, M. J. Physiol. **138**, 449 (1957).
15. ROSSITER, R. J. and NICHOLLS, D. Rev. can. biol. **16**, 249 (1957).
16. FIALA, S., SPROUL, E. E., and FIALA, A. J. Biophys. Biochem. Cytol. **2**, 115 (1956).
17. GOLDZIEHER, J. W., BESCH, P. K., and VELEZ, M. E. J. Biol. Chem. **231**, 445 (1958).
18. SELYE, H. J. Clin. Endocrinol. **6**, 117 (1946).
19. SELLERS, E. A. Rev. can. biol. **16**, 175 (1957).

SEPARATION OF THE VALYL-LEUCYL- AND VALYL-GLUTAMYL-POLYPEPTIDE CHAINS OF HORSE GLOBIN BY FRACTIONAL PRECIPITATION AND COLUMN CHROMATOGRAPHY[1]

STRATHEARN WILSON[2] AND DAVID B. SMITH

Abstract

Horse globin has been fractionated into two components by stepwise addition of acid acetone to an acidified globin solution and by gradient elution with urea from a carboxylic-type ion exchange resin at low pH.

The components obtained by both methods are similar to those prepared earlier by electrophoresis. They have similar molecular weights (about 16,000) and appear to be present in nearly equal amounts. One has the N-terminal sequence valyl-leucyl- and the other valyl-glutamyl-. They differ electrokinetically and in other properties.

Introduction

Reichmann and Colvin (1) showed that horse globin has a number average molecular weight (M_n) of 15,000 to 17,000 and a weight average molecular weight (M_w) of 21,000 to 23,000 in media of low pH and ionic strength. These authors concluded therefore that the horse hemoglobin unit existing in neutral solution and in crystals consists of at least four polypeptide subunits. Electrophoresis of globin under conditions promoting maximum dissociation indicated that the subunits were of two types (1).

Recently these two types of globin subunits have been separated electrophoretically and studies made on the effect of changes in pH and ionic strength on their molecular weights (2, 3). N-Terminal amino acid sequence studies (3) have shown that one of the components has an N-terminal sequence of valyl-leucyl while the other has valyl-glutamyl. Both these sequences had been shown to be N-terminal in horse hemoglobin by Sanger (4).

The present paper describes two further methods of separating the components of horse globin and shows that they are similar to those obtained electrophoretically (2, 3).

Materials and Analytical Methods

Horse oxyhemoglobin and globin were prepared from fresh citrated blood in the manner already described (2).

The N-terminal amino acid of globin and its components was removed by a modification (3) of Edman's phenyl isothiocyanate method (5). Treatment of the protein residue with 1-fluoro-2,4-dinitrobenzene (5) followed by hydrolysis permitted identification of the second amino acid residue. The dinitrophenyl (DNP-) derivatives were separated by paper chromatography using

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[2]National Research Council of Canada Postdoctorate Fellow 1956–58.

either the *tert*-amyl alcohol/phthalate buffer system of Blackburn and Lowther (6) or the two-dimensional system of Levy (7). The spots were eluted into water (7), the solutions clarified by centrifugation, and their optical densities measured at 360 mμ.

A Brice–Speiser Light Scattering Photometer (8) with standard slits and the standard 3 × 3 cm cross-section cells was employed for light scattering measurements. The value of the refractive index increment of globin and its components was taken as 0.200 ml/g at 436 mμ (1). The constant K, in the equation $(Kc/R_{90}) = (1/M_w) + 2Bc$ (9) has therefore the value 6.46×10^{-7} where R_{90} is the reduced intensity of the light scattered at 90°; c, the concentration; M_w, the weight average molecular weight; and B, the second virial coefficient. Dust-free water was obtained by distillation.

Protein (globin or globin component) was dissolved to form an 0.5% solution in pH 1.8 formate buffer (sodium formate 0.04 M, formic acid 2.85 M). This solution was dialyzed, with stirring overnight at 5°, against 20 to 50 volumes of buffer. The solution was clarified by centrifugation at approximately 30,000 r.p.m. for 1.5 hours in a Spinco preparative ultracentrifuge followed by filtration through an ultrafine sintered glass filter. The solvent was clarified by filtration only. After measurement of R_{90} of the buffer, weighed portions of protein solution were added and the value of R_{90} found at each concentration. The concentration of the stock protein solution was measured using a Brice–Speiser differential refractometer.

Two similar portions of protein solution in the pH 1.8 formate buffer were dialyzed with stirring each against 30 volumes of pH 5 formate buffer (sodium formate 0.05 M, formic acid 0.0024 M) changed daily over 3 days at 5°. One of these solutions was taken for the determination of M_w by light scattering while the other was dialyzed to pH 1.8 against the original buffer, prior to light scattering measurements.

The ultraviolet absorption spectra of globin and its fractions (0.1–0.15%) in 0.1 M sodium hydroxide were measured over the range 250 to 350 mμ using a Cary recording spectrophotometer. Corrected values of the optical densities at the two maxima, 283 and 289 mμ, were obtained by subtracting the value at 350 mμ.

Electrophoretic analyses were carried out in a Perkin Elmer Model 38 apparatus.

Fractionation Methods

Fractional Precipitation of Oxyhemoglobin

Oxyhemoglobin (3 g) in 0.5 M sodium carbonate (70 ml) was dialyzed against distilled water (3 × 5 l.) for 24 hours at 0° and then diluted to 125 ml. The precipitation of oxyhemoglobin was investigated in two ways. A portion of the solution (25 ml, pH 8.03) was diluted with water to 100 ml and titrated turbidimetrically. Portions (5 ml) of acetone were added dropwise to the magnetically stirred solution at 22°. After each addition, the optical density of the mixture was measured at 700 mμ using a Beckman spectrophotometer.

The remainder of the oxyhemoglobin solution (100 ml) was fractionally precipitated by the stepwise addition of acetone (20 ml portions) at 5°. The fractions were centrifuged down (30,000 r.p.m., 30 minutes) and washed with 50% v./v. acetone (3 × 20 ml). The heme was removed by dissolving the fractions in 0.02 *M* HCl (10 ml) and adding 90 ml 0.2% HCl acetone (0.2 volumes concentrated HCl in 100 volumes acetone). The globin precipitates were removed by centrifugation (3000 r.p.m., 30 minutes), washed with acetone (3 × 10 ml), and finally dried *in vacuo*. The nature and proportions of the second amino acids were investigated.

Variable Solubility Test on Globin

Globin (550 mg) was dissolved in 0.02 *M* HCl (100 ml). A portion of the solution (1 ml) was removed for determination of the globin concentration using a Brice–Speiser differential refractometer ($\Delta n/c = 0.1925$ ml/g at $\lambda = 578$ mμ (1)). To the remainder of the globin solution, 50 ml of 1.5% acid-acetone (1.5 volumes concentrated HCl in 100 volumes acetone) were added dropwise with magnetic stirring at 5°. The mixture was then allowed to stand for 30 minutes. A suitable volume (5–50 ml) of the supernatant liquid containing 5–10 mg of protein was then withdrawn. The addition of acid-acetone and withdrawal of samples was repeated until all the globin was precipitated (900 ml acid-acetone added).

The globin precipitate was removed from each sample by centrifugation (3000 r.p.m., 10 minutes) and washed with 85% (v./v.) acetone (2 × 5 ml). The combined washings and supernatant liquid were evaporated to dryness at room temperature in a current of air. The residue was washed with acetone (2 × 5 ml) and dissolved in 0.02 *M* HCl (2 ml) for determination of the globin content using the differential refractometer.

Fractional Precipitation of Globin with Acid-Acetone

Globin was fractionally precipitated from a stirred acid solution by the stepwise addition of acid-acetone through a capillary at −30°. The procedure is outlined in Fig. 1. At each step the precipitate was removed by centrifugation and washed with acetone. The major fractions A1 and C3 were fractionated further by a similar process (Figs. 2 and 3).

Chromatographic Fractionation of Globin

The adsorptive capacity of various materials for globin was determined (10) under conditions promoting maximum dissociation (pH < 2, ionic strength < 0.1, and globin concentration < 1.0%) (3). The adsorbent (1 g) was washed with 11.7% (w./v.) formic acid and shaken for 10 minutes with globin (0.1%) in 11.7% formic acid (10 ml). The suspension was clarified by centrifugation and the ultraviolet absorption of the supernatant liquid then measured at 280 mμ in a Beckman spectrophotometer. Among the materials tested were Amberlite CG50 type 2 and CG120 type 2 cation-exchange resins, Dowex 50 × 2 resins, powdered cellulose (Solkafloc), Celite 503, starch, and charcoal. The adsorptive capacity of these materials was less than 2 mg globin per g

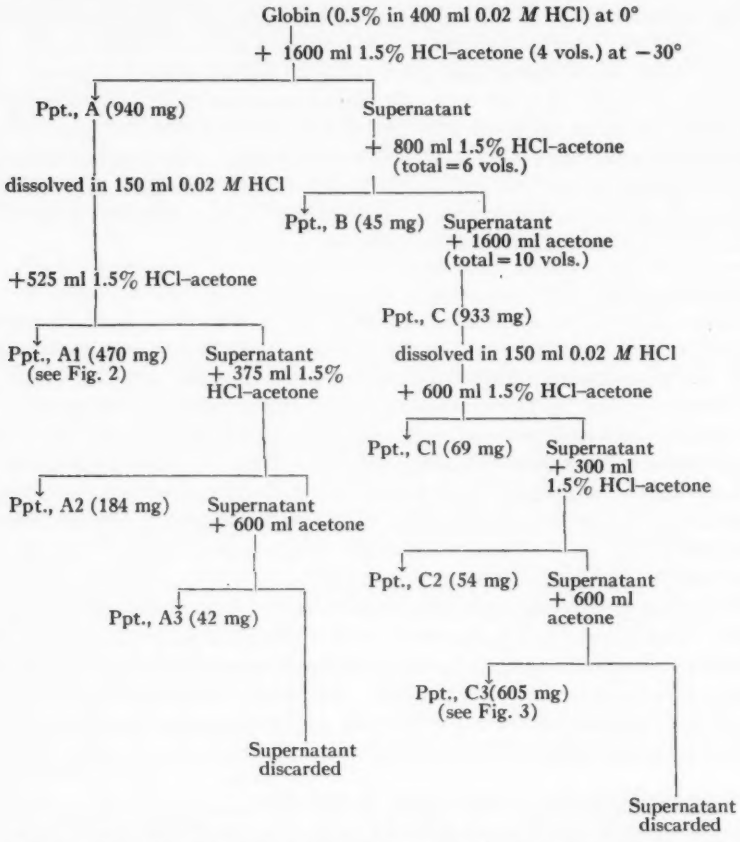
Globin (0.5% in 400 ml 0.02 M HCl) at 0°
+ 1600 ml 1.5% HCl–acetone (4 vols.) at −30°
Ppt., A (940 mg)
Supernatant
+ 800 ml 1.5% HCl–acetone (total = 6 vols.)
dissolved in 150 ml 0.02 M HCl
Ppt., B (45 mg)
Supernatant + 1600 ml acetone (total = 10 vols.)
+525 ml 1.5% HCl–acetone
Ppt., C (933 mg)
Ppt., A1 (470 mg) (see Fig. 2)
Supernatant + 375 ml 1.5% HCl–acetone
dissolved in 150 ml 0.02 M HCl
+ 600 ml 1.5% HCl–acetone
Ppt., C1 (69 mg)
Supernatant + 300 ml 1.5% HCl–acetone
Ppt., A2 (184 mg)
Supernatant + 600 ml acetone
Ppt., C2 (54 mg)
Supernatant + 600 ml acetone
Ppt., A3 (42 mg)
Ppt., C3 (605 mg) (see Fig. 3)
Supernatant discarded
Supernatant discarded

FIG. 1. Flow sheet illustrating fractionation of globin by stepwise addition of acid-acetone.

adsorbent, with the exception of 'Solkafloc' (5 mg/g) and Amberlite CG50 type 2 (carboxylic acid) resin (10 mg/g). Concentrated solutions of urea, guanidine hydrochloride, or acetamide acidified to pH 2 readily removed the adsorbed globin from the 'Solkafloc' and Amberlite CG50 type 2 resin (cf. (11)). The most successful fractionation of globin was obtained by adsorbing the protein on CG50 type 2 resin followed by gradient elution with urea solution (2 to 8 *M*) acidified with HCl to pH 1.9.

The following procedure was found satisfactory. Amberlite CG50 type 2 resin which had been washed with alkali and acid was equilibrated with 11.7% formic acid and poured to form a column (62 × 2.2 cm). Globin was adsorbed from a 1% solution in 11.7% formic acid (70 ml) by a portion (75 g) of the wet resin which was then added to the top of the resin column. The column was washed with 2 *M* urea (1 l.) brought to pH 1.9 with HCl. Gradient elution

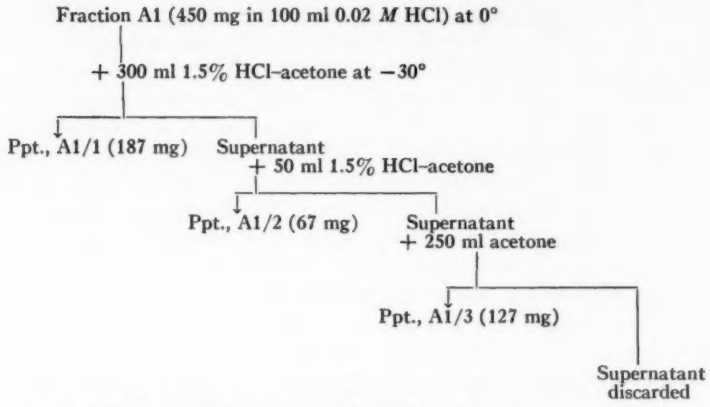
Fraction A1 (450 mg in 100 ml 0.02 M HCl) at 0°
+ 300 ml 1.5% HCl–acetone at −30°
Ppt., A1/1 (187 mg)
Supernatant
+ 50 ml 1.5% HCl–acetone
Ppt., A1/2 (67 mg)
Supernatant
+ 250 ml acetone
Ppt., A1/3 (127 mg)
Supernatant discarded

FIG. 2. Subfractionation of fraction A1 (Fig. 1) by stepwise addition of acid-acetone.

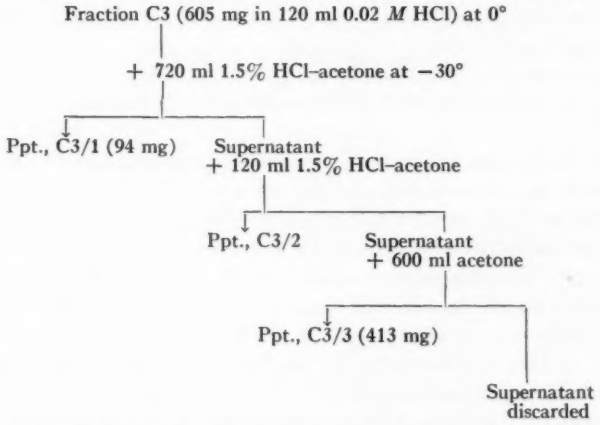
Fraction C3 (605 mg in 120 ml 0.02 M HCl) at 0°
+ 720 ml 1.5% HCl–acetone at −30°
Ppt., C3/1 (94 mg)
Supernatant
+ 120 ml 1.5% HCl–acetone
Ppt., C3/2
Supernatant
+ 600 ml acetone
Ppt., C3/3 (413 mg)
Supernatant discarded

FIG. 3. Subfractionation of fraction C3 (Fig. 1) by stepwise addition of acid-acetone.

was begun by running 8 *M* urea at pH 1.9 into a mixing vessel containing the 2 *M* urea solution (2 l.). The column was washed finally with the 8 *M* urea solution. The flow rate was 2 ml/minute and the temperature was 22°–25°. Twenty-five-milliliter portions of eluate were collected and their optical densities measured at 280 mμ. The eluate was divided into seven fractions which were extensively dialyzed against water (3 days, 6 × 5 l.) at 5° and finally dried at room temperature *in vacuo*.

Results and Discussion

Fractional Precipitation

When horse oxyhemoglobin solution was titrated turbidimetrically with acetone a plot of the optical density at 700 mμ against the volume of nonsolvent added gave a smooth curve with no inflection point. There were no

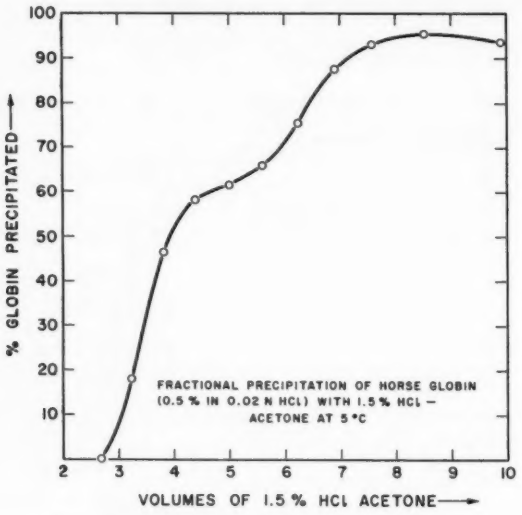

FIG. 4. Precipitation of globin by acid-acetone.

measurable differences in the proportions of the second amino acids in fractions obtained by the stepwise addition of acetone. These findings indicate that it is not possible to separate possible different types of hemoglobins by fractional precipitation with acetone near neutrality.

In contrast, a variable-solvent solubility test on globin at low pH and ionic strength indicated the presence of two components. Figure 4 shows the percentage of globin precipitated from 0.02 *M* HCl solution by acetone containing 1.5% HCl. The point of inflection on the graph when about 60% of the globin has been precipitated indicates that there are at least two types of protein present. The less soluble component precipitates over the range 75–82% of non-solvent whereas the other component requires 84–89% non-solvent.

TABLE I

Ultraviolet absorption and second amino acid assay of precipitated globin fractions

Fraction	$\frac{\epsilon_{283\ m\mu}}{\epsilon_{259\ m\mu}}$ at pH 13	Second amino acid	
		% Leucine	% Glutamic acid
Whole globin	0.996	48	52
A1	0.956	89	11
C3	1.025	14	86
A1/1	0.954	91	9
C3/3	1.025	2	98

Valine was the only N-terminal amino acid found in globin and in the fractions obtained by the stepwise addition of acid-acetone (Figs. 1, 2, and 3). The results of second amino acid analyses and ultraviolet absorption spectra determinations are shown in Table I. Equimolar amounts of DNP-leucine and DNP-glutamic acid were recovered from unfractionated globin. The globin fraction (A1/1), least soluble in acid-acetone, yielded mainly leucine whereas the most soluble fraction (C3/3) had mainly glutamic acid. These results confirm earlier results (3) that horse globin contains two polypeptide components, one having the N-terminal sequence valyl-leucyl-(*vl*-globin) and the other valyl-glutamyl-(*vg*-globin). If the assumption is made that destruction during hydrolysis of the two DNP-amino acids is similar in extent, the two components are present in nearly equal amounts. This point is being investigated further. From the ratio of the extinction coefficients at 283 and 289 mμ ($\epsilon_{283}/\epsilon_{289}$) a greater tyrosine/tryptophane ratio may be inferred (12) for *vl*-globin than for *vg*-globin.

Figure 5 shows representative light scattering results for unfractionated globin. Table II contains values for the light scattering molecular weights of globin and its two components in formate buffer of low pH and ionic strength. The effect of raising and then lowering the pH is shown. It is evident that the three proteins have similar molecular weights (16,000–18,000) at pH 1.8 but

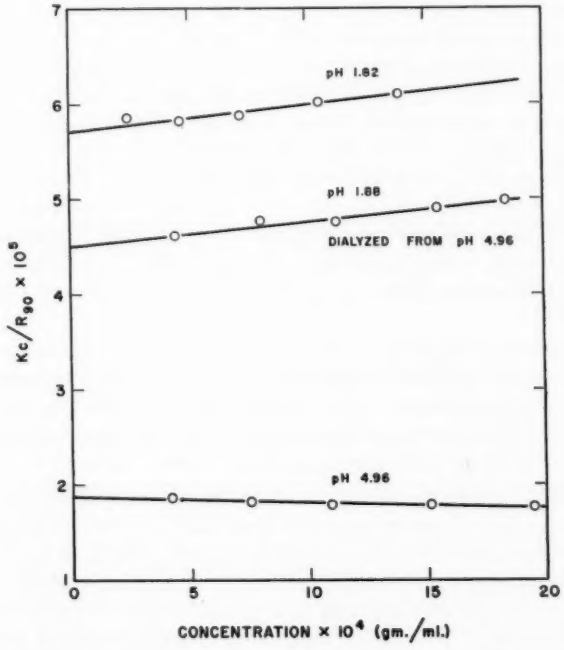

FIG. 5. Light scattering data for unfractionated globin in formate buffers.

TABLE II

The effect of pH changes on the weight-average molecular weight and electrophoretic mobility of globin fractions in formate buffer ($\mu = 0.05$)

Protein	pH	M_w*	B* (ml moles g^{-2})	$10^5 U$ (desc.)† (cm^2 $volts^{-1}$ sec^{-1})
Whole globin	pH 1.82	17,500	0.00135	6.2 and 7.6
	Dialyzed to pH 4.96	53,100	−0.00035	2.7, 4.0, and 5.7
	Dialyzed further to pH 1.88	22,200	0.0014	—
vl-Globin (fraction A1/1)	pH 1.78	16,750	0.00265	6.8
	Dialyzed to pH 4.61	25,300	−0.00058	3.4
	Dialyzed further to pH 1.83	18,600	0.00249	—
vg-Globin (fraction C3/3)	pH 1.78	16,500	0.00204	8.4
	Dialyzed to pH 4.61	69,800	−0.00039	5.8
	Dialyzed further to pH 1.83	30,300	0.00152	—

*Weight average molecular weight (M_w) and second virial coefficient (B) are obtained from the light scattering equation $Kc/R_{90} = 1/M_w + 2Bc$.
†'U' (desc.) is the electrophoretic mobility of the descending protein boundary.

at higher pH values the *vg*-component aggregates to a greater extent than the *vl*-component or unfractionated globin. This aggregation cannot be due to a simple monomer–dimer equilibrium since there is no curvature in the plots of Kc/R_{90} against globin concentration (13). The relatively high value of the second virial coefficient at pH 1.8 reflects the strong Coulombic repulsions between molecules in solutions of low pH (9). These repulsions probably result from the addition of protons to the free amino groups. At pH 4.6, B is small and negative, thus intermolecular Coulombic repulsions become less effective than attractive forces as the pH of the solution is raised and the net positive charge on the protein molecules decreases. These values of B obtained from light scattering measurements in formate buffer at pH 2 are in contrast to studies made in saline media (2) which yielded negligible values for B in the range pH 2–3.5.

The aggregation of *vl*-globin at pH 4.6 is more nearly reversible than that of *vg*-globin when the solution is dialyzed back to pH 1.8. Attempts made to redissociate the *vg*-component by dissolving it in 50% formic acid or 8 M urea solution at pH 2 followed by dialysis against 0.02 M HCl and precipitation with 1.5% HCl–acetone were unsuccessful.

The changes in molecular weight as the pH of the medium is varied are very similar with both these components separated by fractional precipitation and those obtained earlier by electrophoresis (2, 3). In both cases, one component is easily aggregated and the effect is not entirely reversible. The other component is much less affected and the molecular weight changes are nearly completely reversible. It is likely that if the latter component were entirely free of the other, its molecular weight would be constant over the pH range 2–4.

The last column in Table II confirms previous evidence (2) that *vl*-globin is the slower-moving electrophoretic component. Only one peak is evident in the electrophoresis of *vl*- or *vg*-globin at both pH 1.8 and pH 4.6, whereas unfractionated globin yields two and three peaks. The electrophoretic patterns are similar to those shown in a previous report (2). Mixtures of the *vl*- and *vg*-components behave like unfractionated globin. The differences in these electrophoretic mobilities from those of Reichmann and Colvin (1) are probably due to the different buffer system used.

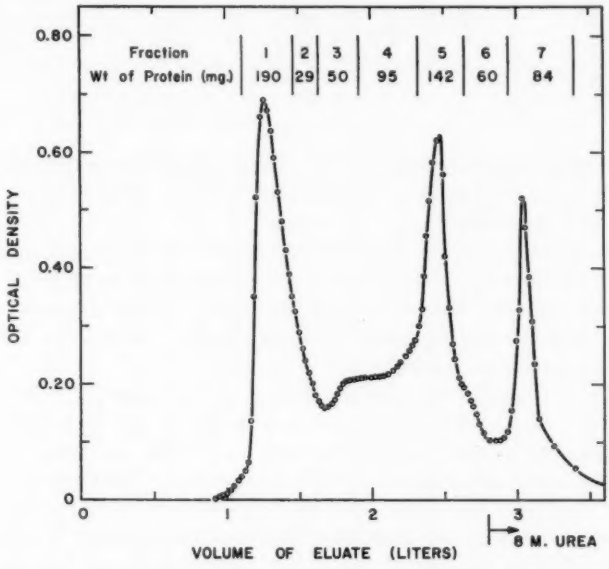

FIG. 6. Elution pattern of globin from a carboxylic acid type ion exchange resin using a gradually increasing urea concentration (2 *M* to 8 *M*) at pH 1.9. Points where cuts between fractions were made and yields are shown.

Chromatographic Fractionation

Figure 6 shows the elution pattern of globin from Amberlite CG50 type 2 resin with urea solution at pH 2. The points where cuts were made between fractions, the fraction numbers, and the amounts recovered in each fraction are indicated in the figure. The total recovery is about 90%. Since analysis of the fractions identified valine as the only N-terminal amino acid, urea treatment does not cause rupture of covalent bonds in the polypeptide chains of globin.

Table III shows that fractions 1 and 2 contain only 3–4% glutamic acid in the second amino acid position whereas fractions 5, 6, and 7 contain only 1–3% of leucine. The first peak in Fig. 6 (fractions 1 and 2) therefore represents almost pure *vl*-globin while the second and third peaks (fractions 5, 6, and 7) represent *vg*-globin of similar purity. Together these two groups account for

TABLE III

Ultraviolet absorption and second amino acid assay of chromatographic fractions

Fraction	$\frac{\epsilon_{283\ m\mu}}{\epsilon_{239\ m\mu}}$ at pH 13	Second amino acid	
		% Leucine	% Glutamic acid
Whole globin	0.999	52	48
Fraction 1	.970	96	4
Fraction 2	.974	97	3
Fraction 3	.997	—	—
Fraction 4	1.008	—	—
Fraction 5	1.016	1	99
Fraction 6	1.018	—	—
Fraction 7	1.025	4	96

72% of the original globin weight. The bimodal elution pattern of *vg*-globin may be related to its tendency to aggregate (2) or it may indicate variation within the *vg*-component.

These results were confirmed by electrophoresis in formate buffer ($\mu = 0.06$) at pH 1.9 and pH 4.6. Fractions 1, 2, 5, 6, and 7 all gave single peaks at both pH's while fractions 3 and 4 gave two peaks. Electrophoresis of a mixture of fractions 1 and 5 in equal amounts yielded a similar pattern to that obtained with unfractionated globin.

The molecular weight by light scattering of the protein in fraction 5 in formate buffer ($\mu = 0.05$) at pH 1.9 was found to be very high (66,700) indicating that the protein was aggregated. A sample was then dissolved in 0.02 *M* HCl and precipitated with 1.5% HCl–acetone. The value of the molecular weight after this treatment (49,000) was still very much higher than that of *vg*-globin obtained by the fractional precipitation method (16,500). It is probable that the *vg*-component would not have aggregated had the dialysis involved 0.01 *M* HCl instead of water. It has been shown (2) that electrophoretically isolated *vg*-globin cannot be removed from a medium of low pH and ionic strength or be freeze-dried without causing irreversible aggregation.

Measurements of the relative heights of the peaks in the ultraviolet absorption spectra of the fractions give values (Table III) which indicate that *vl*-globin has a greater tyrosine/tryptophane ratio than *vg*-globin.

Conclusion

The present work shows that horse globin can be separated into the same two protein components by fractional precipitation and by column chromatography as were obtained previously (2, 3) by electrophoresis. The congruence of the properties of the respective components isolated by the three methods is shown in Table IV. The conclusions of the earlier work that horse globin dissociates into protein subunits of two types each having a monomeric molecular weight of about 16,000 and probably present in nearly equal amounts are confirmed.

TABLE IV

Summary of properties of isolated globin components

	Method of separation					
	Electrophoresis*		Column chromatography		Fractional precipitation	
Fraction	From descending limb	From ascending limb	First eluted	Last eluted	First pptd.	Last pptd.
N-terminal amino acid sequence	Valyl-leucyl	Valyl-glutamyl	Valyl-leucyl	Valyl-glutamyl	Valyl-leucyl	Valyl-glutamyl
$\frac{\text{Tyrosine}}{\text{Tryptophane}}$ ratio	Higher	Lower	Higher	Lower	Higher	Lower
Relative electrophoretic mobility	Slow comp.	Fast comp.	Slow comp.	Fast comp.	Slow comp.	Fast comp.
Effect of increasing pH on molecular weight	Little effect	Aggregates readily	—	—	Little effect	Aggregates readily

*See refs. 2, 3.

The advantage of the new fractionation procedures over the older is the much greater yield. Whereas by electrophoresis only a few milligrams of each component could be obtained readily, the new methods permit a manyfold increase in these quantities.

Nomenclature of Polypeptide Chains in Multichain Proteins

There is at present a lack of agreement on a system for naming the polypeptides in multichain proteins. Sanger (14) designated the two chains derived from insulin as A and B. Rhinesmith, Schroeder, and Martin (15) called the two types of chain shown to exist in human hemoglobin A with the N-terminal sequences valyl-leucyl- and valyl-histidyl-, α and β respectively. Itano and Singer (16) have recently tentatively referred to the chains in human hemoglobin A as "*a*" and "*u*".

An informative designation for these chains would seem to be the one used in this paper where the N-terminal amino acid sequence is indicated by the initial letters of the residues in order. The use of small letters avoids confusion with the systems of capital and Greek letters in use to describe the human hemoglobin complex and the Greek letters and numbers used with serum proteins. The minimum number of residues required to designate each polypeptide chain is two in the cases of horse and human hemoglobins but with most multichain proteins, it is likely to be one. In the relatively few cases likely to occur where the amino acids concerned are either identical or have the same initial letter, numerical subscripts or other indication may also be required. Our suggestion is that a designation for these polypeptide chains need not be largely arbitrary but could also convey useful information.

Acknowledgments

The assistance of Mr. J. L. Labelle is gratefully acknowledged.

References

1. Reichmann, M. E. and Colvin, J. R. Can. J. Chem. **34**, 364 (1956).
2. Haug, A. and Smith, D. B. Can. J. Chem. **35**, 945 (1957).
3. Smith, D. B., Haug, A., and Wilson, S. Federation Proc. **16**, 766 (1957).
4. Sanger, F. Nature, **162**, 491 (1948).
5. Fraenkal-Conrat, H., Harris, J. L., and Levy, A. L. Methods of Biochem. Anal. **2**, 359 (1955).
6. Blackburn, S. and Lowther, A. G. Biochem. J. **48**, 126 (1951).
7. Levy, A. L. Nature, **174**, 126 (1954).
8. Brice, B. A., Halwer, M., and Speiser, R. J. Opt. Soc. Am. **40**, 768 (1950).
9. Doty, P. and Edsall, J. T. Advances in Protein Chem. **6**, 35 (1951).
10. Hirs, C. H. W., Moore, S., and Stein, W. H. J. Biol. Chem. **200**, 493 (1953).
11. Luck, J. M., Cook, H. A., Eldredge, N. T., Haley, M. I., Kupke, D. W., and Rasmussen, P. S. Arch. Biochem. Biophys. **65**, 449 (1956).
12. Beaven, G. H. and Holiday, E. R. Advances in Protein Chem. **7**, 319 (1952).
13. Steiner, R. F. Arch. Biochem. Biophys. **34**, 333 (1952).
14. Sanger, F. Biochem. J. **44**, 596 (1947).
15. Rhinesmith, H. S., Schroeder, W. A., and Martin, N. J. Am. Chem. Soc. **80**, 3358 (1958).
16. Itano, H. A. and Singer, S. J. Proc. Natl. Acad. Sci. U. S. **44**, 522 (1958).

EFFECT OF CENTRALLY ACTING DRUGS UPON MUSCULAR EXERCISE IN RATS[1]

GAÉTAN JASMIN AND PIERRE BOIS

Abstract

By means of a modified revolving-cage test, the effect of methylphenidate as compared with other centrally acting drugs has been studied in rats subjected to forced muscular exercise. Methylphenidate increased the performance of trained animals while amphetamine and ibogaine were inefficient. The running ability of untrained rats was also improved by the administration of methylphenidate. All the central nervous depressants tested (e.g., meprobamate, chlorpromazine, and perphenazine) diminished the performance of animals at the dose utilized. This depressive action was counteracted by methylphenidate. Methylphenidate only antagonized perphenazine when the two agents were given in combination, before the animals were exposed to forced muscular exercise.

With the object of evaluating the effect of centrally acting drugs upon muscular activity in rats, a simple running test has been devised using a revolving cage. The present study deals with the effect of methylphenidate (Ritalin) on the performance of rats subjected to this test. This central stimulant was examined by comparison with other pharmacologic agents of similar activity, amphetamine (Benzedrine) and ibogaine (Lambarene). It was further used as an antagonist against central depressants in the same test.

Methods

Animals

One hundred and twenty female Sprague Dawley rats (Holtzman Farm) weighing between 120 and 130 g were used in these experiments. They were kept on Purina Fox Chow diet and tap water. Animals were selected for experiments according to their ability to run in a revolving cage after a standard training period. All experimental groups were composed of six rats each.

Exercise

The running test was made in a drum-type revolving cage made of a close meshed metallic screen. The drum, 6 ft. in length and 12 in. in diameter, was subdivided into 12 compartments, thus permitting the study of two groups of animals in each test: one experimental and one control. The cage was rotated at 14 r.p.m. by an electric motor operated through a reducing box. Three small hurdles, 1 in. high, were placed at intervals along the runway, so the rats were forced to jump repeatedly during the performance. This addition proved to be most valuable for the objective assessment of the animals' ability.

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Contribution from the Laboratoire de Pathologie expérimentale, Université de Montréal, Montreal, Que. Work done during tenure of Donald McEachern Fellowships of the Muscular Dystrophy Association of Canada and supported in part by a grant No. 604-12-22 from the Ministère de la Santé de la Province de Québec.

When untrained animals are first left in the running cage, they are usually confused and excited by the movement of the apparatus. A few will run readily and keep pace. However, most animals start to jump across the cage and try all kinds of acrobatics. This first contact with the drum must be brief, since during such excitement many animals may hurt themselves. A few days of training will rapidly adapt the rats to the running cage. We adopted a 4-day training schedule which consisted in periods of 15, 20, 30, and 45 minutes each, twice daily, for the first, second, third, and fourth day, respectively. It has been our experience that some animals always run erratically in the cage even after a training period. These were discarded and only good runners were kept. The duration of exercise was set at 3 hours, since it allowed sufficient time to observe significant signs of decreasing performance in the controls. It is worthy of mention here that although untrained rats show abrasions of the paws after a short period, trained animals seldom develop any such lesions even after running for 4 hours.

FIG. 1. Schematic demonstration of the behavior of a rat in a revolving cage while performing exercise. The figures indicate the running capacity according to the position of the rat in the cage.

Readings

The running performance was appraised by direct observation of the animals' behavior in the cage during the test. Readings were recorded according to an arbitrary scale graded from 5 to 0, which corresponds to the running position of the rat in the revolving cage (Fig. 1). Each animal was observed for 15 to 30 seconds in order to determine its grade. Intermediate positions were expressed in fractions of grade. Below 3, grading was made according to the frequency of stumbling and 0 corresponds to the state of complete exhaustion. Repeated observation of animals in the revolving cage has shown us that the running position of a trained rat in the cage is remarkably constant and varies very little for a given time. When starting to run or after treatment with a stimulant they adopt position 5; later, regression will occur and the animals fall back into positions 4 and 3,

keeping pace with the movement of the cage. When a rat starts jumping and stumbling from 4 to 3 in order to avoid running and keeping pace, we consider that it is showing signs of fatigue. The use of hurdles does not permit the animals to slide from one position to another; therefore, the rats are forced into a regular running pattern.

For purposes of comparison, all the results have been expressed as percentages of performance, taking 100% as the animal's ability to maintain the grade 5 position, and are shown in graphic form. The variability in the control group was low as is indicated by the figures given in Table I. As the variability observed in the treated groups did not differ markedly from that of the controls, for the sake of clarity they have not been included in the figures.

Drugs

All drugs were injected subcutaneously in a volume of 0.2 ml. Except for meprobamate, which was given in suspension, they were dissolved in physiologic saline. Dosage was established after many preliminary trials. Essentially these consist in evaluating the optimal dose tolerated by a group of animals all the while keeping their performance within the range of grades 3 to 5 during the first 30 minutes of exposure. The time of treatment is indicated by arrows in each graph.

(1) *Central Nervous Stimulants*

Methylphenidate is a potent cortical stimulant known to exert a very low sympathomimetic action (1). Its effect on muscular exercise was compared with two other drugs: (*a*) ***amphetamine***, a typical central stimulating agent; (*b*) ***ibogaine***, an alkaloid extract obtained from the roots of *Tabernanthe iboga* (an African plant), known to possess some stimulating properties resembling those of strychnine (2) and cocaine (3).

All stimulants were administered at the beginning and during the course of experiment at a dose of 1 mg.

(2) *Central Nervous Depressants*

Phenothiazine derivatives, chlorpromazine (Largactil) and perphenazine (Trilafon), were selected for evaluation under the present experimental conditions. They were given in a single dose of 0.5 mg at the beginning of the experiment. Meprobamate (Equanil), of interest for the purpose of this study as a muscle relaxant, was injected once, at a dose of 10 mg at the time of exposure of the animals to exercise. In experiments in which central nervous stimulants and depressants were given together, the dosage of each remained the same as when the drugs were administered separately.

Results

(1) *Standardization of the Muscular Performance Curve*

In order to establish a reference curve, eight different control groups consisting of six trained rats each were tested for their performance in the

TABLE I

Running capacity of normal trained rats

	Time			
	30 min	1 hour	2 hours	3 hours
Mean values* ± S.E.	3.96 ± .04	3.76 ± .05	3.57 ± .06	3.35 ± .05
Conversion into % (5 = 100)	79	75	71	67

*From readings obtained in eight groups of six rats.

running cage during a 3-hour period. The mean values of the readings taken at fixed intervals (cf. Table I) have been used for the control curve appearing on all graphs.

Table I shows that the optimal performance attained by trained animals after 30 minutes averages 79% of the possible running capacity. Some individuals may perform better, but only for a few minutes at the beginning of the test. This would suggest that animals do not usually perform at their maximal potential. The slight variation in performance which is inevitable at the beginning of the test had usually disappeared after 30 minutes when the first readings were made. The 3-hour test period was found satisfactory, since the imposed muscular effort produced a significant decrease in performance after this period without reaching an exhaustion stage, which would have been more difficult to appraise. The few abrasions seen on the paws of some animals after 3 hours were insufficient to explain a decrease in performance. When untreated rats were kept running for longer periods, their performance usually remained at around 50% until the fifth hour; after this time, great individual variations began to occur and most rats had abrasions on the paws.

(2) *Effect of Central Stimulants*

Both methylphenidate and amphetamine increased the running capacity, as indicated by the 30-minute reading on Fig. 2. However, the stimulating effect of amphetamine soon decreased and a second injection after 1 hour was no longer beneficial. Values for animals treated with methylphenidate remained much above those of the controls during the 3-hour test ($P < .01$) and subsequent studies have shown us that this drug was almost equally effective when given in a single dose of 1 mg. Ibogaine, on the other hand, exhibited no stimulating effect under the present experimental conditions; higher doses even proved to have a depressing action.

It soon became apparent that the animals under the influence of methylphenidate were taking the lead throughout the period of exposure, and probably could have performed at a higher speed of cage rotation. Thus, their high standard seemed related to an acquired alertness and ability. To verify this assumption, we investigated whether methylphenidate could

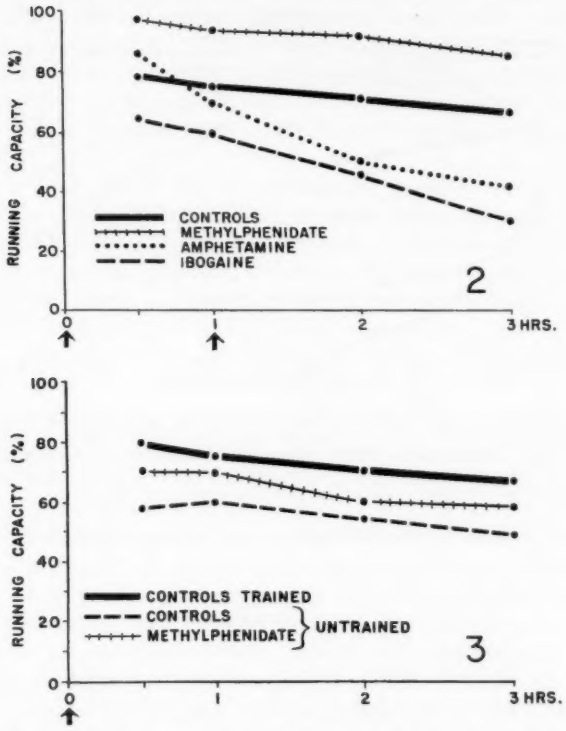

FIG. 2. Effect of central nervous stimulants upon the performance of rats during 3 hours of forced muscular exercise. The heavy line corresponds to values obtained after standardization of muscular fatigue in normal animals.

FIG. 3. Effect of methylphenidate on performance of untrained rats. The running ability is improved though not to the level of trained controls.

enhance the running ability of *untrained rats*. As previously mentioned, when first exposed in the running cage the rats behave awkwardly and their lowered output depends largely on the fact that they rapidly damage their paws. Figure 3 shows that methylphenidate-treated untrained rats definitely run at a higher capacity level than untrained controls ($P = .05$), yet their performance was not as elevated as schedule-trained rats. We may add that their movements were more co-ordinated, as was evidenced by the negligible lesions observed on their paws at the end of the test.

(3) *Effect of Central Depressants*

The depressing agents were given at a dose sufficient to produce sedation without interfering with the normal activity of animals when at rest. It was observed (Fig. 4, left) that all drugs lowered the performance of animals seemingly in proportion to their central inhibitory action. The greater effect of phenothiazines may also be attributed to their influence on the autonomic nervous system.

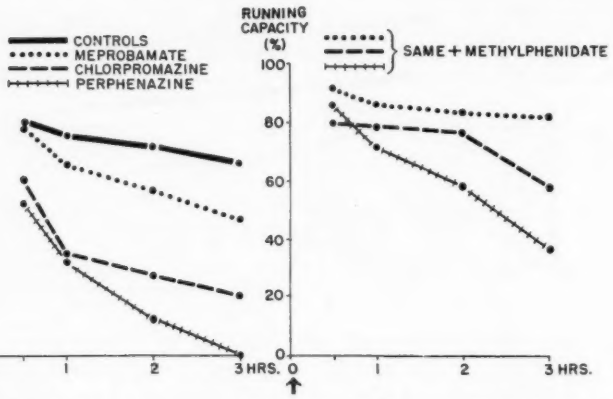

FIG. 4. Effect of central nervous depressants (left) upon the performance of rats and the antagonistic action of methylphenidate on these substances (right). Comparison with the control curve (heavy line, left) shows that the running capacity is lowered by all central depressants. Combined treatment, with methylphenidate, increased performance in proportion to the depressive effect of the drugs.

(4) *Antagonism between Methylphenidate and Depressing Agents*

The antagonistic activity of methylphenidate was found to be relative to the degree of inhibition obtained with central depressants given at the same dose as in the previous test (Fig. 4, right). The central stimulant even raised the running capacity of the meprobamate-treated animals above that of the controls ($P < .02$). Increase in methylphenidate dosage did not further restore the performance of perphenazine-treated rats. Moreover, methylphenidate evidenced no antagonistic effect when administered to perphenazine-treated rats after 1 hour of exercise.

Discussion

The study of factors which influence muscular activity in laboratory animals raises some technical difficulties. In our experience, the effect of drugs on work performance is more readily appreciable by an efficiency test than by an endurance test. In fact, exhaustion is a criterion which is difficult to express in quantitative terms, and we believe that the main interest of our running device is to provide a means for measuring muscular performance.

The present study reveals that centrally acting drugs definitely influence the ability of rats to run in a revolving cage. Treatment with a stimulant, such as methylphenidate, was found to increase the performance of adequately trained rats by almost 20%. Such an improvement may not be explained only by the postponing effect of drugs on muscular fatigue development, since untrained rats likewise benefit from the treatment. We are inclined to believe, in agreement with observations of Meier *et al.* (1), that the drug favors co-ordination of movements. Such a property would be surprising in view of the fact that central nervous stimulants often exert an adverse effect on dexterity (4, 5).

The depressing action of meprobamate was more readily counteracted than that of phenothiazine. This would suggest that the antagonistic property of methylphenidate does not necessarily compensate for the full effect of all depressing agents. Indeed, its stimulating effect was not discernible when the agent was given, after 1 hour of exercise, to perphenazine-treated animals.

By comparison of studies made on isolated muscles, our running test provides us with a more adequate evaluation of the effects of drugs on muscular activity. The experiments reported here do not claim to evaluate all factors contributing to muscular exertion; however, the results obtained suggest that certain central nervous stimulants may prove of interest in promoting muscular work and favoring co-ordination of movements.

Acknowledgments

The authors wish to thank Doctor Pierre Biron for his technical assistance. They are also indebted to Ciba Co., Montreal; Poulenc Ltée, Montreal; Purdue Frederick Co., New York; Schering Corp., Montreal; Smith, Kline & French Laboratories, Montreal; and Wyeth Laboratories, Montreal, who have graciously supplied the Ritalin, Largactil, Lambarene, Trilafon, Benzedrine, and Equanil.

References

1. MEIER, R., GROSS, F., and TRIPOD, J. Klin. Wochschr. **32**, 445 (1954).
2. SCHNEIDER, J. A. and SIGG, E. B. Ann. N. Y. Acad. Sci. **66**, 765 (1957).
3. RAYMOND-HAMET, Compt. rend. soc. biol. **133**, 426 (1940).
4. HORST, K., ROBINSON, W. D., JENKINS, W. L., and BAO, D.-L. J. Pharmacol. Exptl. Therap. **52**, 307 (1934).
5. GOODMAN, L. S. and GILMAN, A. The pharmacological basis of therapeutics. 2nd ed. The Macmillan Co., New York. 1956. p. 518.

ESTIMATION OF THYROXINE OUTPUT BY THE THYROID GLANDS OF NORMAL AND ADRENALECTOMIZED RATS BY MEANS OF A SIMPLE COOLING TEST[1]

MELVIN J. FREGLY

Abstract

Adrenalectomy, administration of propylthiouracil (PTU), or a combination of these two treatments significantly increased rate of cooling of rats restrained and subjected to air at 5° C. Propylthiouracil administration increased rate of cooling 55% above that of untreated control rats while adrenalectomy increased rate of cooling 84%. The combination of these two treatments increased rate of cooling 94%. Dead rats cooled 201% faster than control rats. Administration of graded doses of thyroxine to both adrenalectomized PTU-treated rats and to normal PTU-treated rats indicated that 1.9 and 4.4 μg/day of thyroxine respectively was required to return rate of cooling to that of comparable controls. These doses are therefore best estimates of daily thyroxine outputs by thyroid glands of these animals. The results suggest that adrenalectomy is accompanied by diminished thyroid function.

Many separate studies have shown that rats without adrenals survive only a few hours when subjected to cold air (1–3). Surgically thyroidectomized rats may survive 4 or 5 days at similar temperatures (1, 4). Despite these facts, little is known of the relative contributions of each of these glands to the maintenance of body temperature of rats subjected to cold air. Previous studies had shown that adrenalectomy increased rate of cooling of rats subjected to cold air approximately 66% above that of normal rats subjected to cold air in the same fashion (3). An objective of the experiments reported here was to determine the effect of thyroidectomy and of thyroidectomy plus adrenalectomy on cooling rate in order to assess the relative contributions of each organ to maintenance of body temperature during exposure to cold air. It also seemed possible that this simple cooling procedure might be used to estimate daily output of thyroxine by thyroid glands of normal and adrenalectomized rats. The results of the experiment support this possibility and suggest that the thyroxine output of adrenalectomized rats is less than half that of normal rats.

Methods and Results

Male rats of the Holtzman strain were used in all experiments. Body weights of the rats ranged from 200 to 250 g. This range was strictly maintained because of the known effects of body weight on cooling rate (3). Large rats have been shown to cool more slowly than smaller ones.

Three separate experiments were performed. The materials and methods which were common to all three experiments are described below. The rats

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were kept two or three per cage in a room maintained at 24 ± 1° C and illuminated from 8 A.M. to 6 P.M. All rats were given Purina laboratory chow as food and were allowed ad libitum choice between tap water and 0.15 *M* NaCl solution as drinking fluids until the cooling test took place.

One week before the cooling test, the adrenals of the rats were removed. The operation was performed by the dorsal approach while the rats were under ether anesthesia. Control rats were sham operated to assess the effects of the operation. Sham operation consisted of removal of a piece of fatty tissue from the region of the adrenal gland.

To effect chemical "thyroidectomy", propylthiouracil[2] (PTU), at a level of 0.1%, was mixed thoroughly into the diet. Treatment was begun 2 weeks prior to the cooling test.

Colonic temperature was measured using a copper–constantan thermocouple which was inserted 5 cm into the colon of the rat. The thermocouples were led off to a potentiometer which recorded colonic temperature continuously. The rats were restrained prior to being cooled by taping each foot with adhesive tape and leaving a free tab which was tacked to a board. After restraint for 15 or 20 minutes at room temperature, the rats were placed in a thermoregulated room maintained at 5 ± 2° C. The relative humidity of the room varied from 80 to 85%. Each rat was kept in cold air until its colonic temperature fell to 26° C, at which time it was removed from the cold, freed, and rewarmed artificially. Since repeated exposures to air at 5° C have been shown to adapt rats to cold (i.e., reduce rate of cooling), each rat in these experiments was used in only one test (5). Over the range of colonic temperatures from 38 to 26° C, the relationship between colonic temperature and duration of exposure to cold air was found in previous studies to be linear for both sham-operated and adrenalectomized rats (3, 5). Therefore, colonic cooling rate (CCR) measured in degrees Centigrade per hour was subsequently used to assess response of rats to cold. Statistical analysis of the difference between means was performed by the use of the *t* test for the 95% confidence limit (6).

Experiment 1

One hundred and three rats were used in this experiment. Thirty-two rats were given propylthiouracil (PTU); 21 were adrenalectomized; 14 were treated with PTU *and* adrenalectomized; 7 were sacrificed by ether inhalation just prior to the cooling experiment; and 29 were sham-operated controls.

The results of this experiment are given in Table I. In the order of increasing cooling rates the groups may be arranged as follows: sham-operated, PTU-treated, adrenalectomized, adrenalectomized + PTU-treated, and dead rats. Thus, PTU treatment increased colonic cooling rate (CCR) 54.9% above that of control rats; adrenalectomy, 84.0%; adrenalectomy + PTU treatment, 99.2%; while dead rats cooled 200.7% faster than control rats. It appears that the effects of adrenalectomy and PTU-treatment

[2]6-*n*-Propyl-2-thiouracil, Nutritional Biochemicals Co., Cleveland, Ohio.

TABLE I

The effect of thyroid and adrenal glands on colonic cooling rate and resting colonic temperature of rats

Group	Number of rats	Mean body weight, g	Resting colonic temp., ° C	Colonic cooling rate, ° C/hr	P*	% increase above control group
Sham-operated	29	248	37.4±0.1†	3.93±0.31†	—	—
PTU-treated	32	224	36.5±0.2	6.09±0.28	<.01	54.9
Adrenalectomy	21	238	36.7±0.2	7.23±0.26	<.01	84.0
Adx + PTU	14	215	34.9±0.3	7.83±0.27	<.01	99.2
Dead	7	252	36.3±0.3	11.82±0.52	<.01	200.7

*Probability value (comparison with CCR of sham-operated group).
†Standard error of the mean.

are not quantitatively additive. Treatment of adrenalectomized rats with PTU did not increase CCR significantly above that of adrenalectomized untreated rats ($P = 0.14$). Each experimental group listed in Table I cooled significantly ($P<0.01$) faster than the sham-operated, control rats.

Table I also lists the initial mean colonic temperature of each group of rats in air at 25° C. Each of the treatments listed reduced resting colonic temperature significantly ($P<0.01$) below that of control rats.

Experiment 2

Forty-four rats were used in this experiment. Fifteen rats were treated with PTU for 2 weeks prior to the cooling test and were given an intraperitoneal injection of 2.5 μg thyroxine[3]/day (in 0.25 ml normal saline) for 2 days before and on the day of the cooling test (1 hour before cold exposure). Nine other rats were also treated with PTU but were given 3 μg thyroxine/day as above while a third PTU-treated group of 8 rats received 4 μg thyroxine/day. Control rats were injected with 0.25 ml saline. A fifth group of 12 rats was given PTU plus 0.2% desiccated thyroid powder,[4] mixed thoroughly into the diet, for 2 weeks preceding the test. All rats were subjected to the standard cooling test described above.

The results of this experiment are shown in Table II and Fig. 1. Administration of 2.5 μg thyroxine/day to PTU-treated rats did not counteract completely the effect of propylthiouracil on colonic cooling rate. These rats cooled 39.6% faster than sham-operated controls. Administration of 3 μg thyroxine/day to PTU-treated rats returned CCR to only 12.7% above that of control rats while administration of 4 μg returned rate of cooling nearly to normal (+7.6%). Differences between mean CCR's of sham-operated rats and rats receiving 3 or 4 μg thyroxine were not significant. Addition of 0.2% desiccated thyroid powder to the food appeared to produce an effect on CCR equivalent to daily injection of 3.2 μg thyroxine/day.

[3]Squibb thyroxine fraction.
[4]Armour thyroid powder No. 58, U.S.P.

TABLE II

The effects of propylthiouracil and thyroxine on colonic cooling rate and resting colonic temperature of sham-operated rats

Group	Number of rats	Mean body weight, g	Resting colonic temp., ° C	Colonic cooling rate, ° C/hr	P*	% increase above control group
Sham-operated	29	248	37.4±0.1†	3.93±0.31†	—	—
PTU-treated	32	224	36.5±0.2	6.09±0.28	<.01	54.9
PTU + 2.5 μg thyroxine	15	231	37.4±0.1	5.49±0.25	<.01	39.6
PTU + 3.0 μg thyroxine	9	241	38.1±0.1	4.43±0.56	.44	12.7
PTU + 4.0 μg thyroxine	8	243	38.3±0.1	4.23±0.44	.64	7.6
PTU + 0.2% desiccated thyroid powder	12	234	37.9±0.2	4.92±0.25	.06	25.2

*Probability value (comparison with CCR of sham-operated group).
†Standard error of the mean.

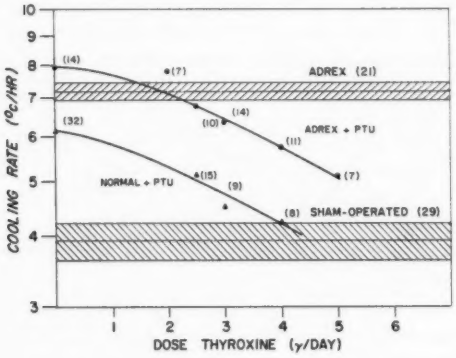


FIG. 1. The logarithm of colonic cooling rate (° C/hr) graphed against the dose of thyroxine administered (μg/day) to adrenalectomized (●) and normal (▲) rats receiving PTU. The mean colonic cooling rate of adrenalectomized untreated rats, ±1 standard error, is shown as a line across the top of the figure; that for sham-operated rats is represented by a line across the bottom of the figure. The numbers in parentheses indicate the numbers of rats used.

Administration of even the lowest dose of thyroxine (2.5 μg /day) returned to normal the initial resting colonic temperature measured prior to cooling (Table II). Further increase in dose level to 3 and 4 μg/day caused an elevation in colonic temperature significantly above that of sham-operated rats.

It may be observed in Fig. 1 that a smooth curve connecting the mean cooling rates of PTU-treated, normal rats given 0, 2.5, 3, or 4 μg thyroxine/day would cross the line representing mean cooling rate of sham-operated

rats at 4.4 μg thyroxine/day. If it is assumed that PTU completely inhibits only thyroid function, this value would appear to be an estimate of the daily thyroxine output of the thyroid gland of the normal rat since it is this dose of thyroxine which returns rate of cooling of PTU-treated rats to that of untreated, normal rats.

Experiment 3

Sixty-three rats were used in this experiment. All rats were adrenalectomized 1 week prior to the cooling test. When PTU was used, treatment was begun 2 weeks prior to the cooling test while thyroxine treatment was begun 2 days prior to the cooling test with the final dose being given on the day of the cooling test, 1 hour before cold exposure. The various treatments, number of rats in each group, mean body weight, initial body temperature prior to cold exposure, and colonic cooling rate are given in Table III. The probability values shown in Table III indicate that treatment with PTU and 4 or 5 μg thyroxine/day significantly decreased CCR.

In Fig. 1 it may be observed that 1.9 μg thyroxine/day returned CCR of PTU-treated, adrenalectomized rats to that of adrenalectomized rats. Higher doses of thyroxine decreased rate of cooling still further. Administration of 5.0 μg/day of thyroxine to PTU-treated, adrenalectomized rats reduced CCR significantly below that of adrenalectomized rats and to within 29% of that of sham-operated rats.

Table III indicates that administration of 3.0 μg or more of thyroxine/day to adrenalectomized PTU-treated rats returned resting body temperature to

TABLE III

The effects of propylthiouracil and thyroxine on the colonic cooling rate and resting colonic temperature of adrenalectomized rats

Group	Number of rats	Mean body weight, g	Resting colonic temp., °C	Colonic cooling rate, °C/hr	P*	P†
Sham-operated	29	248	37.4±0.1‡	3.93±0.31‡	—	—
Adrenalectomized	21	238	36.7±0.2	7.23±0.26	—	<.01
Adx. + PTU	14	215	34.9±0.3	7.83±0.27	.14	<.01
Adx. + PTU + 2 μg thyroxine	7	253	36.3±0.3	7.81±0.61	.32	<.01
Adx. + PTU + 2.5 μg thyroxine	10	231	35.7±0.1	6.77±0.35	.41	<.01
Adx. + PTU + 3.0 μg thyroxine	14	241	37.3±0.2	6.36±0.41	.07	<.01
Adx. + PTU + 4.0 μg thyroxine	11	243	37.5±0.2	5.77±0.41	<.01	<.01
Adx. + PTU + 5.0 μg thyroxine	7	223	37.3±0.2	5.08±0.19	<.01	.09

*Probability value (comparison of CCR with that of adrenalectomized group).
†Probability value (comparison of CCR with that of sham-operated group).
‡Standard error of the mean.

that of sham-operated rats. Lower doses were ineffective in this regard. None of the doses used increased the body temperature of adrenalectomized rats above that of sham-operated rats as was observed for normal PTU-treated rats (Table II).

Discussion

The rate of fall of colonic temperature of restrained rats exposed to air at 5° C is influenced both by adrenal and thyroid glands. Removal of either increases rate of cooling. It is interesting that the combination of adrenalectomy with propylthiouracil treatment did not make the effects of each additive. The contribution to heat production by muscular activity of adrenalectomized rats may be different from that of thyroidectomized or normal rats. This possibility could help to explain the differences in cooling rates of these three groups of rats. Ring (7) has shown that total body heat production of adrenalectomized rats at any given body temperature during cooling was less than that of normal rats. It is likely that the lower metabolism of adrenalectomized rats during cooling is due either to muscular fatigue (less shivering) or to depletion of carbohydrate stores or both. Other experimental evidence exists which supports the fact that muscles of adrenalectomized rats fatigue more quickly than those of normal rats (8). The relative fatigability of thyroidectomized rats apparently has not been tested.

These experiments suggest that adrenalectomy, in addition to abolishing adrenal function, also appears to diminish thyroid function. Administration of PTU to adrenalectomized rats did not increase significantly cooling rate above that of adrenalectomized rats (Table III). Measurement of cooling rates of PTU-treated adrenalectomized rats given graded doses of thyroxine suggests that the daily output of thyroxine by adrenalectomized rats is less than half that of normal rats. This interpretation of the data agrees with the findings of Flückiger and Verzár (9), who observed that tracer doses of radioactive iodine were picked up normally by thyroid glands of adrenalectomized rats but were released much more slowly. These authors suggested that the decrease in release of radioactive iodine by the thyroid gland following adrenalectomy was a consequence of the reduced metabolic rate accompanying this condition. The reduced thyroid function of adrenalectomized rats is an important consideration in attempting to assess the relative contributions of adrenal glands and thyroid gland to maintenance of body temperature in the cold. This finding suggests another possible explanation for the faster cooling of adrenalectomized rats as compared with PTU-treated rats.

The estimated thyroxine output of the thyroid gland of normal rats in this experiment was 4.4 μg/day. This value is similar to that observed by Dempsey and Astwood (10) (5.2 μg/day), Reineke *et al.* (11) (4.8 μg/day), and Woods and Carlson (12) (10 μg *d,l*-thyroxine/day) to be necessary for reduction of thyroid size to normal after treatment with thiouracil and maintenance at an air temperature of 25° C. Similar experiments were not performed by these investigators on adrenalectomized rats.

It is interesting to compare the data of experiment 1 with those observed in experiments 2 and 3. The combination of adrenalectomy and thyroidectomy increased rate of cooling 94% above that of sham-operated, control rats, while thyroidectomy (PTU treatment) alone increased cooling rate 55% above that of controls. The difference between these two should be due to lack of adrenal glands (39%). But adrenalectomy alone was observed to increase rate of cooling 84% above that of control rats. The difference between this value and that calculated by difference above (39%) must be that resulting from lack of thyroid function (45%). If it is assumed that PTU treatment causes complete inhibition of thyroxine formation by the thyroid gland, then the thyroid gland of adrenalectomized rats must be functioning at $(55-45)/55\times100$ or 19% of its normal activity. Assuming a normal daily thyroxine output of 4.4 μg/day, the cooling rate data above would suggest that the daily thyroxine output of adrenalectomized rats was about 0.8 μg/day. This is about half of that calculated from experiment 3 (1.9 μg/day). Qualitatively, this rough calculation also suggests a decreased thyroid function in adrenalectomized rats. The quantitative differences between the daily output of thyroxine calculated above and that observed in experiment 3 may be due to the implicit assumption used above, viz., that thyroidectomy inhibits only thyroid function and adrenalectomy inhibits only adrenal function. This assumption is probably incorrect. For example, experiment 3 has demonstrated that adrenalectomy is accompanied by decreased thyroid function. On the other hand, surgical thyroidectomy (13) or treatment with *thiouracil* (14–16) appear to produce adrenal atrophy and diminished adrenal function, although propylthiouracil does not (17, 18).

It may be observed in Fig. 1 that linear extrapolation of the curve for adrenalectomized rats treated with PTU to the line representing the mean colonic cooling rate of sham-operated rats would give an intersection at approximately 7.1 μg thyroxine/day. This is the amount of thyroxine required theoretically to return rate of cooling of PTU-treated adrenalectomized rats to that of sham-operated rats. This value is considerably higher than that necessary to return rate of cooling of PTU-treated normal rats to that of sham-operated rats (4.4 μg/day). It is possible that this difference in dose levels between adrenalectomized and sham-operated rats may represent a difference in utilization of thyroxine at the cellular level. Indeed, Hoffman, Hoffman, and Talesnik (19) observed that the oxygen consumption of adrenalectomized–thyroidectomized rats given thyroxine did not increase by the same percentage as that of thyroidectomized rats given the same dose of thyroxine. Administration of adrenal cortical extract to the adrenalectomized–thyroidectomized rats resulted in a normal response to injections of thyroxine.

Although it has been observed that adrenalectomized rats cool faster than thyroidectomized rats, it cannot be stated unequivocally that adrenal glands are more important in maintenance of body temperature during cold exposure under these conditions. The more rapid cooling rate of adrenalectomized

rats could be due to: (*a*) decreased thyroid function; (*b*) decreased utilization of thyroxine at the cellular level; (*c*) decreased muscular activity and shivering; (*d*) a combination of all these factors; and (*e*) other factors. Conditions (*a*) and (*b*) have been observed experimentally; the other possibilities must await further experimentation.

Acknowledgment

I thank Mr. W. Selser for technical assistance.

References

1. SELLERS, E. A., YOU, S. S., and THOMAS, N. Am. J. Physiol. **165**, 481 (1951).
2. SELYE, H. and SCHENKER, V. Proc. Soc. Exptl. Biol. Med. **39**, 518 (1938).
3. IAMPIETRO, P. F., FREGLY, M. J., and BUSKIRK, E. R. Can. J. Biochem. and Physiol. **34**, 721 (1956).
4. LEBLOND, C. P. and GROSS, J. Endocrinology, **33**, 155 (1943).
5. FREGLY, M. J. Am. J. Physiol. **173**, 393 (1953).
6. FISHER, R. A. Statistical methods for research workers. 10th ed. Hafner Publishing Co., N. Y. 1948. p. 114.
7. RING, G. C. Am. J. Physiol. **122**, 435 (1938).
8. INGLE, D. J. Am. J. Med. **19**, 724 (1955).
9. FLÜCKIGER, E. and VERZÁR, F. J. Endocrinol. **13**, 39 (1955).
10. DEMPSEY, E. W. and ASTWOOD, E. B. Endocrinology, **32**, 509 (1943).
11. REINEKE, E. P., MIXNER, J. P., and TURNER, C. W. Endocrinology, **36**, 64 (1945).
12. WOODS, R. and CARLSON, L. D. Endocrinology, **59**, 323 (1956).
13. FREEDMAN, H. H. and GORDON, A. S. Proc. Soc. Exptl. Biol. Med. **75**, 729 (1950).
14. BAUMAN, E. J. and MARINE, D. Endocrinology, **36**, 400 (1945).
15. DEANE, H. W. and GREEP, R. O. Endocrinology, **41**, 243 (1947).
16. ZARROW, M. X. and MONEY, W. L. Endocrinology, **44**, 345 (1949).
17. GABRILOVE, J. L. and SOFFER, L. J. Endocrinology, **47**, 461 (1950).
18. PERRY, W. F. Can. J. Med. Sci. **30**, 36 (1952).
19. HOFFMAN, F., HOFFMAN, E. J., and TALESNIK, J. J. Physiol. **107**, 251 (1948).

IMPAIRMENT OF TUMOR GROWTH BY UNNATURAL AMINO ACIDS[1]

FERNAND MARTEL AND LOUIS BERLINGUET

Abstract

Fourteen unnatural amino acids were assayed for their action on the development of a transplantable Novikoff hepatoma tumor, and 10 of them were found to exert some inhibitory effects.

Introduction

The growth-inhibitory effects of specific metabolite antagonists have been demonstrated, the most notable of them being related to purines and pyrimidines. So far, only a few amino acid antagonists have been found. Due to their structure, these substances are apt to play a decisive role in the protein metabolism of rapidly proliferative tissue such as cancerous tumor.

The present work was undertaken in an attempt to detect possible effects of some non-natural amino acids on cancer growth by testing them on the development of a Novikoff hepatoma in the rat.

Though this tumor is not generally recognized as a choice material for chemotherapeutic studies, it has been found useful to use it as a preliminary step to more elaborate and more systematic chemotherapeutic assays. It has also been found to lend itself well to the appraisal of metabolic changes, as was pointed out by the studies of Goranson *et al.* (11, 12) on the effect of alloxan diabetes on tumor growth impairment.

Materials and Methods

Among the amino acids tried, six can be related structurally to glycine, in which the alpha-carbon is substituted: 1-amino, 1-carboxylic cyclopentane acid (I); 1-amino, 1-carboxylic cyclohexane acid (II); 1-ethylamino, 1-carboxylic cyclohexane acid (III); 1-methylamino, 1-carboxylic cyclohexane acid (IV); 2-methylamino, 2-phenylacetic acid (V), and 2-amino, 2-phenylacetic acid (VI). They have been synthesized in our laboratory (1).

Two unnatural amino acids which were tested are related to serine by having an hydroxyl radical on the carbon chain: 2-amino, 5-hydroxy valeric acid (VII), and 2-amino, 6-hydroxy caproic acid (VIII). Both have been synthesized for the first time in our laboratories (2, 3), and have interesting biochemical properties (8, 9, 16, 13, 14, 15, 7). One of them (VIII) has been found to be an antagonist to lysine in the diet of the rat (17, 18).

Two higher homologues of methionine were also tested: 2-amino, 5-mercapto valeric acid (XI), and 2-amino, 6-mercapto caproic acid (XIV) (4).

[1]Manuscript received in original form March 27, 1958, and, as revised, October 2, 1958. Contribution from the Department of Biochemistry, School of Medicine, Laval University, Quebec, Que. This work was supported by research grants from the National Cancer Institute.

Two substances were related to phenylalanine: N-methyl-phenylalanine (XII) (5) and phenylalaninol, or 2-amino, 3-phenyl, propanol-1 (XIII) (6).

Finally, 2-amino, 2-methyl propionic acid (X), a substituted alanine, and 2-amino, 4-pentenoic acid, or allyl-glycine (IX) were selected for trial. The last one has already been found to act as an antimetabolite of methionine (10). All the amino acids tested were of the DL form.

Parenteral solutions of these amino acids were prepared by dissolution in sterile saline (NaCl 0.85%). Titers of these solutions were so adjusted that the desired amount to be injected daily, per 100 g of body weight, would be contained in 1 ml.

Male rats of the Wistar strain, obtained from commercial stock, and weighing between 125 and 200 g were used. Purina Fox Chow and water were available to the animals ad libitum.

Groups of animals were injected, intraperitoneally, with a saline suspension of a 7-day-old Novikoff hepatoma.[2] Suspensions were prepared by forced passage of an aseptically excised tumor through a No. 18 hypodermic needle and subsequent transfer to a measured volume of NaCl 0.85% to obtain a 1/10th dilution of the tumor. After agitation, this suspension was injected through a No. 20 needle. Amounts of 1 ml were injected once per rat.

Intraperitoneal injections of amino acids were made daily, for 7 days, starting with the day of transplantation. Control rats, bearing transplants, received 1 ml of NaCl 0.85% per 100 g of body weight, per day.

Body weights were recorded at the beginning and at the end of the 7-day experimental period. On the 7th day, rats were killed by decapitation, and tumors were dissected out and weighed in the wet state.

It has been a fact of current observation that the hepatoma tumor grows most of the time in a diffuse way. This constitutes a certain difficulty in the complete dissecting out of the whole growing mass. However, after careful examination and sufficient experience, the authors have been able to ascertain the usual emplacements of tumor growth. There is an important mass of cancerous tissue located at the bottom of the stomach and adherent to the stomach itself, the spleen, and the upper part of the intestinal loops. This mass accounts for more than four-fifths of the whole tumor, and can be dissected out easily. Other cancerous islets are located among intestinal viscera and can be distinguished by their macroscopic appearance from surrounding fat tissue or mesentery.

Results

In Table I are given the results of three consecutive experiments, with recorded death rate, percentages of tumor takes on surviving animals, and weights of tumors.

[2]We are indebted to Dr. Arthur W. Ham of the Ontario Cancer Institute for the original Novikoff hepatoma transplant.

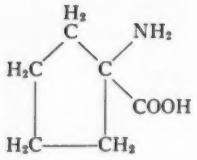
TABLE I

Effects of 14 unnatural amino acids on the growth of a Novikoff hepatoma tumor in rats

Substances tried	No. rats	Initial body wt., g	Body wt. gain or loss	Daily dose (mg/kg of body wt.)	Death rate	Rate of tumor takes in surviving animals	Mean tumor wt., g	S.E. of mean differences	Value of "t" (as compared with NaCl-treated rats)
				First experiment					
NaCl	8	148	+27	85	0/8	7/8	3.6	0.67	
V	10	144	+17	100	1/10	7/9	2.8	0.77	0.8
VI	10	144	+25	10	4/10	5/6	1.9	1.09	1.3
VII	10	146	+21	100	2/10	6/8	2.2	0.65	1.4
VIII	10	143	+21	100	1/10	7/9	1.5	0.47	2.4
IX	10	141	—	100	10/10	—	—	—	—
X	10	142	−14	100	1/10	8/9	1.9	0.39	2.7
XI	10	144	+24	100	4/10	3/6	1.2	0.58	2.6
XII	10	142	+20	100	2/10	5/8	1.5	0.68	2.2
XIII	10	144	−3	100	5/10	4/5	2.6	1.35	0.6
XIV	10	139	+10	20	0/10	7/10	1.7	0.54	2.2
				Second experiment					
NaCl	8	190	+23	85	0/8	8/8	8.0	0.86	
I	10	193	−22	100	0/10	10/10	3.2	0.56	4.7
II	10	192	+19	100	0/10	7/10	2.5	0.82	4.6
III	10	187	+24	100	2/10	5/8	3.6	1.55	2.5
IV	10	177	+18	100	1/10	8/9	3.5	1.51	2.6
IX	6	154	−11	25	1/6	5/5	3.0	0.87	2.7
IX	6	165	−11	50	1/6	4/5	5.3	1.57	1.1
XI	6	157	−2	100	0/6	6/6	4.5	0.97	1.8
XII	6	163	−6	40	1/6	4/5	2.7	1.09	2.7
XIV	6	165	−8	40	1/6	4/5	3.7	2.02	1.6
				Third experiment					
NaCl	5	164	+6	85	0/5	5/5	5.0	0.90	
XIV	15	162	−5	40	0/15	13/15	2.2	0.51	2.7

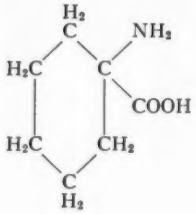
Unnatural amino acids used for screening in cancer chemotherapy

I



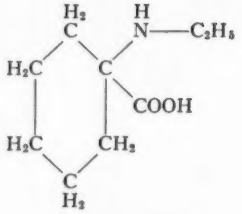
1-Aminocyclopentane-1-carboxylic acid

II



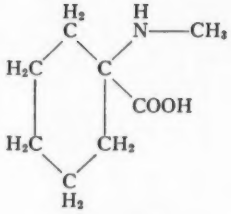
1-Aminocyclohexane-1-carboxylic acid

III



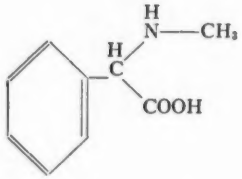
1-(N-Ethylamino) cyclohexane-1-carboxylic acid

IV



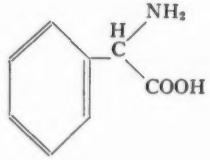
1-(N-Methylamino) cyclohexane-1-carboxylic acid

V



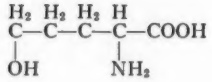
N-Methyl-2-phenylglycine

VI



2-Phenylglycine

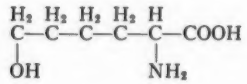
VII



5-Hydroxynorvaline (2-amino-5-hydroxy-valeric acid)

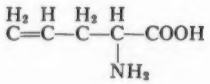
Unnatural amino acids used for screening in cancer chemotherapy

VIII



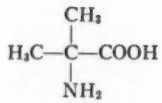
6-Hydroxynorleucine
(2-amino-6-hydroxy-caproic acid)

IX



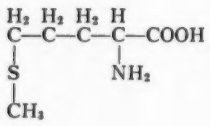
Allyl-glycine

X



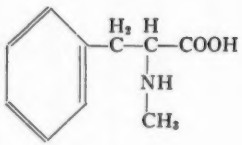
Methylalanine
(2-amino-2-methyl-propionic acid)

XI



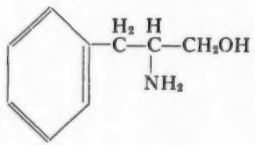
5-(Methylthio)norvaline
2-amino-5(methylthio)-
valeric acid

XII



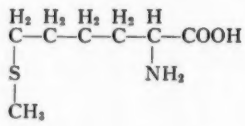
N-Methylamino
phenylalanine

XIII



Phenylalaninol

XIV



6-(Methylthio)norleucine
2-amino-6(methylthio)-
caproic acid

Body weights were affected in different ways by the various amino acids, but as a general observation, it is apparent that no relation exists between weight loss or gain, and death rate, percentage of tumor takes, or tumor weights.

Most substances were first tried at daily doses of 100 mg/kg of body weight, unless earlier tests had indicated that a substance would be highly toxic at this level.

Results of the first experiment show that substances VIII, X, XI, XII, and XIV significantly reduce tumor growth. But, with the exception of XI and XII, they have no important effect on tumor-take rates. Substances V, VI, VII, and XIII do not impair tumour-growth. In the same experiment, substance IX was found to be very toxic at the dose of 100 mg/kg of body weight, causing death of all the animals tested.

From the second experiment, it is seen that tumor-growth impairment can occur with amino acids I, II, III, IV, IX, and XII. This experiment did not confirm, however, the results obtained with XI in the preceding one. The only difference in the experimental procedure was in the number of animals, which was smaller in the second than in the first experiment; the lower "t" value may thus be explained.

Substance IX was tried at two different levels (25, and 50 mg/kg), and it happened that the lowest level was the most effective in reducing tumor growth.

Substance XIV did not repeat its effects as observed in the first experiment. Therefore, a third experiment was done to ascertain if this amino acid would give more significant values on a larger number of animals. It happened effectively that with 15 rats instead of 6, the tumor-growth reducing effect of this substance attained a significant "t" value.[3]

Discussion and Conclusion

In view of its usual diffuse mode of growth in the peritoneal cavity, and the apparent difficulty of its dissection, the Novikoff hepatoma has not been used frequently in chemotherapeutic assays. However, the authors are of the opinion that with proper handling by skilled technicians, this tumor may help in the performance of preliminary assays of potential chemotherapeutic agents in small laboratories where systematic screening tests cannot be performed.

The results of such studies must be taken as an indication of the action of a compound or a group of compounds rather than as an absolute sign of their effectiveness.

With such considerations in mind, the authors feel right to conclude that 10 of the unnatural amino acids tried (I, II, III, IV, VIII, IX, X, XI, XII, and XIV) possess chemotherapeutic properties on cancer by the fact that they have been able to reduce growth of a Novikoff hepatoma in the rat.

[3]While these assays were being performed, complete screening tests were conducted on the same substances by the Cancer Chemotherapy National Service Center. Unofficial reports received from Dr. Howard W. Bond indicate antitumor activity with substance No. I and preliminary encouraging results with substance IX.

Among these, four are related structurally to glycine (I, II, III, and IV), two are related to methionine (XI, and XIV), one to phenylalanine (XII), and one is a known antimetabolite of methionine (IX).

Acknowledgments

We gratefully acknowledge the technical assistance of Mr. André Matte.

References

1. BERLINGUET, L. Unpublished results.
2. GAUDRY, R. Can. J. Research, B, **26**, 387 (1948).
3. GAUDRY, R. Can. J. Chem. **29**, 544 (1951).
4. GAUDRY, R., BERLINGUET, L., GINGRAS, R., and MARTEL, F. Rev. can. biol. **11**, 132 (1952).
5. BERLINGUET, L. Unpublished results.
6. BERLINGUET, L. Can. J. Chem. **32**, 31 (1954).
7. GAUDRY, R. and MARTEL, F. Compt. rend. soc. biol. **148**, 472 (1954).
8. GINGRAS, R., PAGÉ, E., and GAUDRY, R. Science, **105**, 621 (1957).
9. GINGRAS, R. and PAGÉ, E. Rev. can. biol. **6**, 801 (1957).
10. GOODMAN, I., KATSURA, S., and DITTMER, K. Arch. Biochem. **20**, 95 (1949).
11. GORANSON, E. S., BOTHAM, F., and WILLMS, M. Cancer Research, **14**, 730 (1954).
12. GORANSON, E. S. and TILSER, G. J. Cancer Research, **15**, 626 (1955).
13. MARTEL, F. and GINGRAS, R. Rev. can. biol. **9**, 199 (1950).
14. MARTEL, F., GAUDRY, R., and GINGRAS, R. Rev. can. biol. **10**, 246 (1951).
15. MARTEL, F. Can. J. Biochem. and Physiol. **35**, 655 (1957).
16. MERTZ, E. T., BEESON, W. M., WALTZ, R. H., JR., and GAUDRY, R. Proc. Soc. Exptl. Biol. Med. **69**, 609 (1948).
17. PAGÉ, E., GAUDRY, R., and GINGRAS, R. J. Biol. Chem. **171**, 831 (1947).
18. PAGÉ, E., GINGRAS, R., and GAUDRY, R. Can. J. Research, E, **27**, 264 (1949).

LE DOSAGE DE L'ADRÉNALINE ET DE LA NORADRÉNALINE

I. RÉACTION MODIFIÉE DES CATÉCHOLAMINES AVEC L'ÉTHYLÈNEDIAMINE[1]

GUY NADEAU ET GEORGES SOBOLEWSKI

Abstract

A modification to the method of Weil-Malherbe and Bone for the estimation of adrenaline and noradrenaline is suggested. The catecholamines are first allowed to react with ammonium molybdate at room temperature. Ethylenediamine reagent is then added to the catecholamine–molybdate complexes, and the reaction is carried out in two different steps: at room temperature, and at 50° C. This procedure ensures a more adequate oxidation of the catecholamines and controls the variable effect of metallic ions either present in biologic samples or introduced during adsorption on alumina. The fluorescence is also significantly increased, although the amount of ethylenediamine added is reduced, thus decreasing the fluorescence of the reagent blanks. Another modification of the original method for the differential estimation of both catecholamines is based solely on the different rates of reaction of the molybdate complexes with ethylenediamine; this eliminates the need of reading the fluorescence at two different wavelengths.

Introduction

La méthode de dosage de l'adrénaline et de la noradrénaline dans les liquides biologiques, telle que proposée par Weil-Malherbe et Bone (1, 2), comporte essentiellement trois étapes: (*a*) la séparation des catécholamines par chromatographie sur alumine, (*b*) leur réaction avec l'éthylènediamine pour obtenir un matériel fluorescent extractible par l'isobutanol et (*c*) la mesure de la fluorescence de l'échantillon et d'un blanc, à deux longueurs d'onde distinctes (où chacune des catécholamines contribue à la fluorescence totale en proportion différente).

Cette méthode, d'un intérêt incontestable, a cependant soulevé de nombreuses discussions et critiques. La reproduction des résultats semble particulièrement difficile, sinon impossible; la spécificité elle-même est sérieusement mise en doute. Elle offre pourtant de précieux avantages: la fluorescence obtenue à partir de très petites quantités de catécholamines est très intense (à comparer avec la méthode fluorimétrique de Lund (3) aussi couramment employée); elle varie de façon strictement linéaire avec la teneur en catécholamines pour des solutions pures; elle s'additionne bien pour les deux amines et, de plus, elle demeure stable pendant plusieurs heures. Cette dernière particularité constitue un avantage important sur la méthode de Lund, en particulier pour les dosages effectués en série.

Nous nous sommes efforcés de retracer l'origine des faiblesses de la méthode de Weil-Malherbe et Bone et d'y remédier. Nous avons pu en rattacher les difficultés (*a*) à la purification chromatographique des catécholamines, (*b*)

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aux propriétés de la réaction catécholamines–éthylènediamine et (*c*) à la manière de différentier la fluorescence des deux amines au moyen de deux filtres secondaires.

La difficulté de *reproduction* des résultats vient de la réaction elle-même qui fournit des valeurs de fluorescence variables pour une même quantité d'adrénaline (ou de noradrénaline), à cause de facteurs non contrôlables (tels que la présence d'ions métalliques).

Le manque de *spécificité* vient de ce que le blanc, utilisé pour apprécier la fluorescence nette des catécholamines, est inadéquat. En effet, les quantités respectives d'adrénaline et de noradrénaline se calculent en tenant compte du rapport de la fluorescence mesurée à 450 et à 580 mμ. Ce rapport n'est pas nécessairement le même pour une solution des réactifs dans l'eau distillée que pour des extraits de plasma, par exemple.

Nous avons pu apporter à la méthode originale des modifications essentielles qui éliminent ces inconvénients, tout au moins en grande partie. Dans la présente communication, nous discutons des changements apportés à la réaction entre les catécholamines et l'éthylènediamine et à la manière de différentier l'adrénaline de la noradrénaline dans un mélange. Dans une publication subséquente, nous présenterons une étude du problème des blancs de plasma, des modifications que nous proposons, ainsi que de leur application aux dosages biologiques.

Partie expérimentale

Réactifs et appareillage

Adrénaline.—Solution concentrée (10 mg/litre) de la base libre (E.K. 3097) dans l'acide acétique à 5%, conservée à la glacière. Des dilutions dans l'eau distillée sont préparées quotidiennement.

Noradrénaline.—Solution concentrée et dilutions de bitartrate de lévartérénol cristallisé ("Levophed" de Winthrop-Stearns of Canada) préparées comme les précédentes.

Réactif molybdique.—Solution de molybdate d'ammonium c.p. à 1% dans l'eau distillée.

Éthylènediamine.—C.P. fraîchement distillée.

Acide chlorhydrique.—Azéotropique fraîchement distillé.

Isobutanol.—C.P. fraîchement distillé.

La pureté des solvants et des solutions est systématiquement vérifiée au fluorimètre. Les solutions contaminées sont remplacées par des solutions fraîches.

Le fluorimètre utilisé consiste en une modification du spectrophotomètre Beckman, modèle B, que nous avons décrite ailleurs (4). Le filtre primaire est une combinaison des filtres Corning 3389 et 5113 (5-74) transmettant la raie 436 mμ du mercure; le filtre secondaire, un filtre jaune Corning 3486 (3-69).

La verrerie employée est réservée exclusivement à cet usage. Elle est lavée avec un bon détergent et rincée plusieurs fois avec de l'eau distillée.

Réaction modifiée avec l'éthylènediamine

1. *Principe*

Les inconvénients à la base de la méthode originale découlent d'une oxydation mal contrôlée des catécholamines qui précède la condensation avec l'éthylènediamine. La vitesse et le degré de cette oxydation dépendent de la quantité d'oxygène disponible en solution et sont facilement influencés par des traces d'ions métalliques divers. Certains chercheurs (5, 6), avant nous, avaient noté une augmentation de la fluorescence, quand ils appliquaient la réaction à des éluats d'alumine, sans soupçonner que les ions Al^{+++} (et probablement certaines impuretés métalliques) de la colonne en étaient responsables. C'est ce que nous avons démontré dans une communication antérieure (7). Il faut ajouter toutefois que cette augmentation de fluorescence s'évalue difficilement (tableau I), car elle dépend de l'alumine dont la pureté peut être très variable d'un lot à l'autre. Notons enfin que certains investigateurs (8) ont tenté de tirer profit de cet effet, en vue de compenser les pertes rencontrées à d'autres étapes de la technique. Le procédé se passe de commentaires.

Dans le présent travail, nous proposons à la méthode de Weil-Malherbe et Bone une modification qui a l'avantage d'éliminer la difficulté que l'on vient de mentionner. Elle consiste à faire réagir l'adrénaline (et la noradrénaline)

TABLEAU I

Influence de l'alumine sur la fluorescence de l'adrénaline et de la noradrénaline

Auteurs	Date	Pourcentage d'augmentation de fluorescence	
		Adrénaline	Noradrénaline
Valk et Price (5)	Mars 1956	50%	—
Mangan et Mason (6)	Juin 1957	30%	9%
Mangan et Mason (8)	Août 1957	20 à 30%	Moins de 4%
Nadeau, Joly et Sobolewski (7)	Avril 1958	30%	—

TABLEAU II

Comparaison de la fluorescence obtenue par la méthode de Weil-Malherbe et Bone et par la méthode proposée

Catécholamine	Solvant (acide acétique)	Réactif molybdique	Fluorescence finale de l'extrait*	
			à 21° C	à 50° C
Adrénaline (1 μg)	Pur	—	8.5	26.0
Adrénaline (1 μg)	Passé sur alumine	—	23.0	32.0
Adrénaline (1 μg)	Pur	1.5 ml	35.5	37.5
Adrénaline (1 μg)	Passé sur alumine	1.5 ml	37.5	37.5
Noradrénaline (1 μg)	Pur	—	1.5	7.5
Noradrénaline (1 μg)	Passé sur alumine	—	3.5	11.5
Noradrénaline (1 μg)	Pur	1.5 ml	6.0	7.5
Noradrénaline (1 μg)	Passé sur alumine	1.5 ml	8.5	11.0

*Unités arbitraires.

d'abord avec le molybdate d'ammonium et à traiter tel que le complexe molybdique par l'éthylènediamine. Cette façon de procéder présente plusieurs avantages:

(*a*) Elle amène à un niveau constant l'augmentation autrement incontrôlable de la fluorescence due aux impuretés métalliques (tableau II).

(*b*) Elle augmente de façon sensible la fluorescence formée par l'éthylènediamine. On peut ainsi constater, dans le tableau II, que la fluorescence est passée de 26.0 unités (conditions de Weil-Malherbe et Bone) à 37.5 unités (avec le réactif molybdique à 50° C) pour 1 microgramme d'adrénaline.

(*c*) Elle permet de réduire sensiblement la quantité d'éthylènediamine et de diminuer ainsi le blanc des réactifs.

(*d*) Elle peut enfin servir de base à un dosage différentiel d'un principe différent de celui de la méthode originale.

2. Réaction préliminaire entre catécholamines et molybdate

Comme critère des conditions les meilleures au cours de cette réaction, nous nous sommes appuyés sur l'intensité de fluorescence produite par l'action subséquente de l'éthylènediamine, selon la technique décrite plus loin.

L'effet de la quantité de molybdate employé dans la réaction préliminaire est illustré dans la figure 1. L'addition d'environ 0.6 ml de réactif molybdique par 10 ml de milieu réactionnel donne le maximum de fluorescence qui devient indépendante de l'augmentation de réactif.

La réaction est complète au bout de 30 minutes environ à la température ambiante (21° C). La figure 2 illustre l'effet de la température (21° et 50° C) et de la durée de la réaction adrénaline–molybdate sur la fluorescence obtenue après réaction avec l'éthylènediamine. On remarque qu'une fois la réaction complétée entre l'adrénaline et le molybdate, la même intensité de fluorescence s'obtient dans les deux cas.

3. Réaction du complexe molybdique avec l'éthylènediamine

Nous avons adopté au départ des conditions analogues à celles décrites par Weil-Malherbe et Bone (1), sauf pour les détails suivants: (*a*) Le réactif (EDA) est préparé en portant un mélange de 60 ml d'éthylènediamine et de 30.8 ml d'acide chlorhydrique azéotropique (tous deux fraichement distillés) à 100 ml avec de l'eau distillée. Ce mode préparatoire donne sensiblement la même composition que celle du réactif utilisé dans la méthode originale. (*b*) L'extraction par l'isobutanol est effectuée en omettant le relargage par le chlorure de sodium qui contient constamment des impuretés fluorescentes, en dépit de cristallisations répétées dans l'eau distillée.

La quantité de réactif EDA nécessaire pour obtenir le maximum de fluorescence est indiquée dans la figure 3. L'intensité de la fluorescence demeure constante à partir de 0.4 ml. À noter qu'il faut 1.5 ml du même réactif pour obtenir un maximum de fluorescence, si l'on omet la réaction préliminaire avec le molybdate.

La réaction avec l'éthylènediamine effectuée à 50° C ne donne pas l'effet maximum que l'on peut obtenir en procédant en deux étapes: d'abord à la

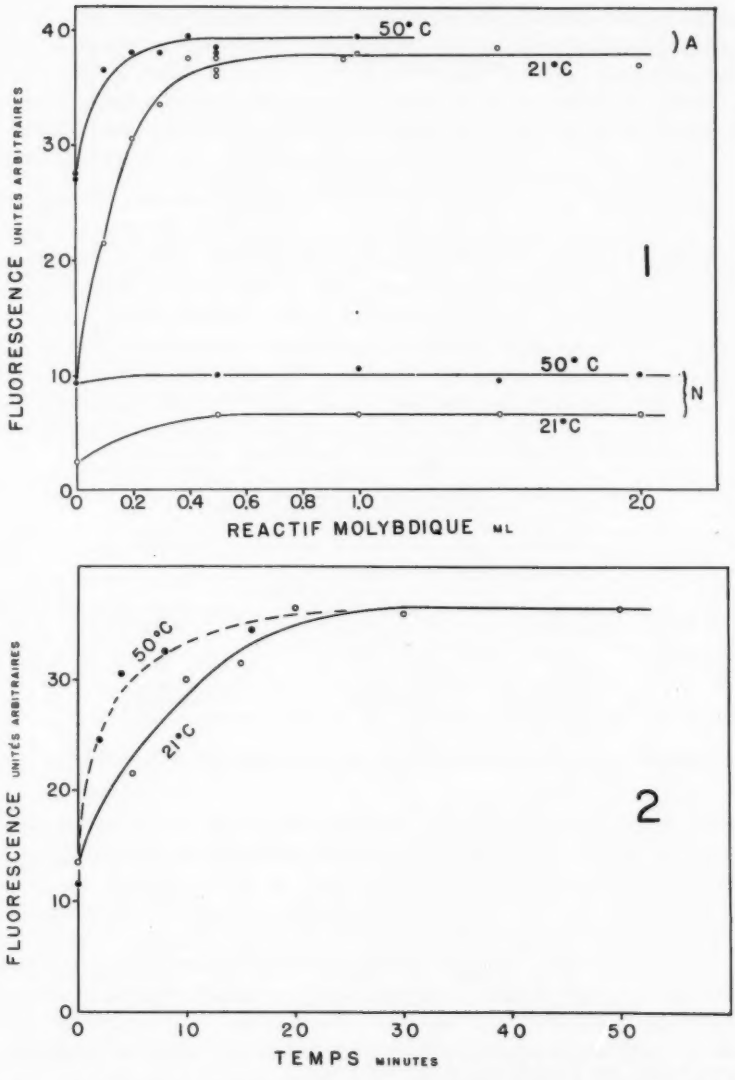
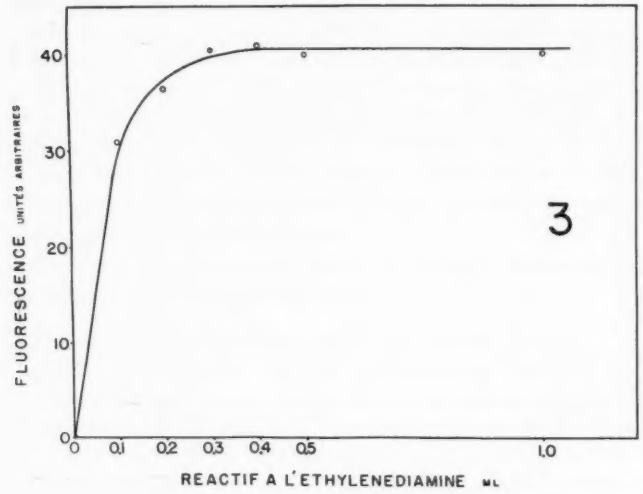


FIG. 1. Influence de la quantité de réactif molybdique sur la fluorescence de l'adrénaline (A) et de la noradrénaline (N) après réaction à la température ambiante et à 50° C.
FIG. 2. Effet de la durée de la réaction entre l'adrénaline et le molybdate d'ammonium sur la fluorescence finale de l'extrait.



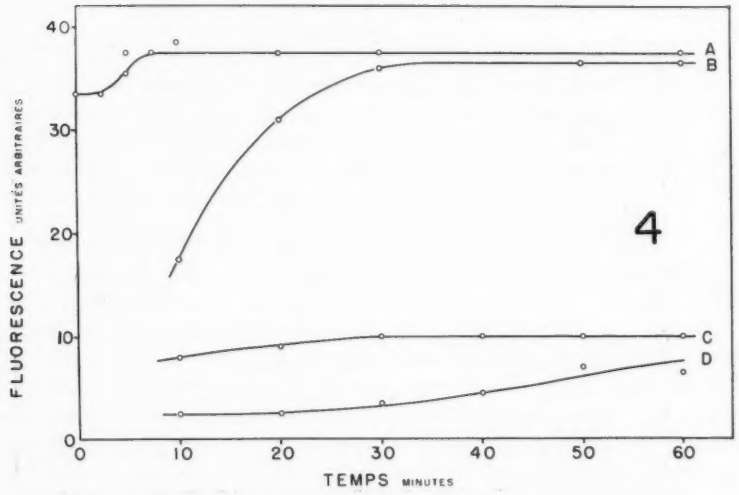


FIG. 3. Influence de la quantité de réactif à l'éthylènediamine sur la fluorescence obtenue à partir du complexe adrénaline–molybdate.

FIG. 4. Effet de la durée de la réaction entre l'éthylènediamine et les complexes molybdiques de l'adrénaline (A et B) et de la noradrénaline (C et D) à la température ambiante (B et D) suivie d'un chauffage de 30 minutes à 50° C (A et C).

température ambiante, puis à 50° C. L'influence de la durée de la réaction à froid (suivie d'un chauffage de 30 minutes à 50° C) est représentée dans la figure 4. On y voit qu'une période d'au moins 10 minutes à froid est nécessaire pour obtenir la fluorescence maximum. Le fait de prolonger cette période n'affecte plus la fluorescence. Il est à noter que le même phénomène existe même en l'absence de molybdate. Il est cependant moins net et, même après une heure de réaction à la température ambiante, le maximum de fluorescence n'est pas encore atteint (résultats non publiés).

Étude de la réaction d'oxydation

Dans une série d'expériences, nous avons effectué la réaction à la fois selon la méthode originale de Weil-Malherbe et Bone et selon la modification proposée dans le présent travail, dans des atmosphères d'azote, d'oxygène ou d'air libre. L'atmosphère était contrôlée jusqu'au stage d'extraction par l'isobutanol inclusivement. Les résultats de cette étude sont compilés au tableau III. Ils confirment que la présence d'oxygène est nécessaire dans le milieu réactionnel et indiquent que la réaction est incomplète à l'air seul, mais devient plus adéquate en présence de molybdate d'ammonium.

TABLEAU III

Influence de l'oxygène sur la réaction entre l'adrénaline et l'éthylènediamine

Atmosphère de réaction	Fluorescence finale de l'extrait	
	Weil-Malherbe et Bone	Modification proposée
Azote	2.0	2.0
Air libre	63.0	85.0
Oxygène	90.0	89.0

Dosage différentiel de l'adrénaline et de la noradrénaline dans un mélange

1. Principe

Dans le dosage des catécholamines par l'éthylènediamine, deux méthodes ont été proposées pour différentier l'adrénaline de la noradrénaline. La méthode de Weil-Malherbe et Bone (2) s'appuie sur la différence des propriétés spectrales; la seconde, celle de Manger *et al.* (9), tire profit de l'action sélective de l'hyposulfite de sodium sur la noradrénaline. Cette dernière ne semble pas toutefois avoir attiré l'attention d'autres chercheurs.

Les deux méthodes ont leurs inconvénients. Celle de Manger *et al.* oblige à diviser les échantillons de façon à effectuer deux réactions parallèles et rappelle un des inconvénients de la méthode de Lund (3). Quant à la procédure suggérée par Weil-Malherbe et Bone, sa valeur a été mise en doute dans les dosages du plasma, à cause de l'interférence de substances possédant des propriétés spectrales analogues à l'une ou l'autre catécholamine (5).

Nous proposons une méthode basée sur un principe différent. En effet, les complexes molybdiques obtenus par l'action du molybdate d'ammonium sur l'une et l'autre catécholamine, réagissent à des vitesses différentes avec

l'éthylènediamine à la température de la chambre. Après 30 minutes de réaction, le pourcentage de transformation est de 96% pour l'adrénaline, mais seulement de 43% pour la noradrénaline. En conséquence, deux mesures de fluorescence, l'une effectuée après 30 minutes de réaction à la température ambiante, et l'autre, une fois la réaction complétée, permettent d'évaluer les quantités respectives des deux amines.

2. Suggestion de mode opératoire

À la solution de catécholamines (10 ml), on ajoute 0.8 ml du réactif molybdique et on abandonne le mélange pour 30 minutes à la température ambiante et à l'obscurité. (La lumière possède, en effet, une certaine influence sur cette réaction.) On ajoute 0.5 ml du réactif à l'éthylènediamine et on laisse le mélange pour une autre période de 30 minutes à la température ambiante et à l'obscurité. (Cette réaction est aussi photocatalysée (10).) On extrait par l'isobutanol (10 ml dans notre cas) et on mesure la fluorescence (F') à une seule longueur d'onde (filtre jaune Corning 3486). On retourne l'extrait alcoolique dans le mélange réactionnel et on chauffe à 50° C pour 30 minutes. On refroidit, on agite les deux phases et on mesure de nouveau la fluorescence (F'') de la couche alcoolique. (Remarque: Si l'on a affaire à une seule catécholamine, la méthode peut être simplifiée comme suit: après la réaction avec le molybdate et l'addition du réactif à l'éthylènediamine, le mélange est laissé à la température de la chambre pour 10 minutes et porté à 50° C pour 30 minutes. On extrait et on mesure la fluorescence.)

3. Équations et résultats

À 30 minutes, aussi bien qu'à la fin de la réaction, la fluorescence totale, moins la fluorescence du blanc, est égale à la somme des fluorescences de l'adrénaline et de la noradrénaline,
soient:

F' : fluorescence mesurée après 30 minutes de réaction avec l'éthylènediamine à la température ambiante,
F'' : fluorescence mesurée à la fin de la réaction,
F_{totale} : fluorescence totale de l'échantillon,
F_{blanc} : fluorescence du blanc,
F_{corr} : fluorescence corrigée,
F_{A} : fluorescence due à l'adrénaline,
F_{N} : fluorescence due à la noradrénaline,
Q_{A} et Q_{N} : quantités respectives d'adrénaline et de noradrénaline.

On a:

$$(a) \quad F'_{\text{totale}} - F'_{\text{blanc}} = F'_{\text{corr}} = (Q_{\text{A}} \times F'_{\text{A}}) + (Q_{\text{N}} \times F'_{\text{N}})$$

et

$$(b) \quad F''_{\text{totale}} - F''_{\text{blanc}} = F''_{\text{corr}} = (Q_{\text{A}} \times F''_{\text{A}}) + (Q_{\text{N}} \times F''_{\text{N}}).$$

Pour des raisons de commodité, on peut régler la sensibilité du fluorimètre, de sorte qu'une solution étalon d'adrénaline accompagnant les échantillons donne une lecture constante de 100 sur l'échelle. Ainsi $F'_{\text{A}} \equiv F''_{\text{A}}$ et les équations (a) et (b) donnent des solutions ayant la forme suivante:

$$Q_{\text{A}} = K_1 (F'_{\text{corr}} - K_2 F''_{\text{corr}}),$$
$$Q_{\text{N}} = K_3 (F''_{\text{corr}} - F'_{\text{corr}}),$$

où K_1, K_2 et K_3 représentent des coefficients déterminés expérimentalement pour chaque appareil. La valeur K_2 est donnée par la relation suivante:

$$K_2 = F'_N/F''_N.$$

À titre d'exemple, à partir d'une série de mesures effectuées avec des quantités d'adrénaline variant de 0.01 à 0.04 microgrammes et de noradrénaline variant de 0.1 à 0.3 microgrammes, nous avons établi les équations suivantes:

$$Q_A = 0.00181\ (F'_{corr} - 0.413\ F''_{corr}),$$
$$Q_N = 0.0105\ (F''_{corr} - F'_{corr}).$$

Le tableau IV compare les quantités connues d'un mélange des deux catécholamines avec les valeurs calculées au moyen des équations précédentes.

TABLEAU IV

Résultats de l'analyse de mélanges d'adrénaline et de noradrénaline

Échantillon	Fluorescence*		Adrénaline ($\times 10^{-2}\ \mu g$)		Noradrénaline ($\times 10^{-1}\ \mu g$)		Pourcentage d'erreur	
	Après 30 minutes	À la fin de la réaction	Ajoutée	Trouvée	Ajoutée	Trouvée	Adr.	Noradr.
1	19.5	31.5	1.00	0.87	1.00	1.10	−13.0	+10.0
2	30.0	42.0	2.00	1.98	1.00	1.10	− 1.0	+10.0
3	47.5	59.5	4.00	3.82	1.00	1.10	− 4.5	+10.0
4	35.0	53.5	2.00	2.01	2.00	1.78	+ 0.5	−11.0
5	57.0	75.0	4.00	4.39	2.00	1.74	+10.0	−13.0
6	44.0	73.5	2.00	2.16	3.00	2.95	+ 8.0	− 1.6
7	60.5	91.5	4.00	3.81	3.00	3.11	− 5.0	+ 3.6
Blanc	4.0	5.5						

*Unités arbitraires.

Discussion

Dans une adaptation de la méthode de Weil-Malherbe et Bone au dosage de la noradrénaline dans les préparations pharmaceutiques, Erne et Canbäck (11) préconisent l'emploi de molybdate d'ammonium comme catalyseur favorable à la formation du dérivé fluorescent. Ce sel est incorporé au réactif à l'éthylènediamine lui-même, i.e. en milieu alcalin dans lequel le complexe adrénaline–molybdate est instable. Conséquemment l'action du molybdate y est peu efficace. Malgré son intérêt, la modification ne semble pas avoir retenu l'attention d'autres chercheurs.

Il est bien établi que la réaction entre l'éthylènediamine et l'adrénaline (et la noradrénaline) procède par plusieurs étapes dont la première consiste en une oxydation de la catécholamine. Cette oxydation, favorisée par le milieu alcalin, s'effectue grâce à l'oxygène dissous. C'est l'étape la plus difficile à contrôler. En effet, il faut tenir compte d'au moins deux facteurs: (*a*) la quantité d'oxygène disponible et (*b*) la sensibilité de la réaction vis-à-vis de l'action catalytique des ions métalliques.

La quantité d'oxygène nécessaire dépend évidemment de la teneur des solutions en catécholamines. Il semble que les concentrations d'adrénaline et de noradrénaline rencontrées dans les liquides biologiques nécessitent justement des quantités d'oxygène voisines de sa limite de solubilité, ce qui peut expliquer en partie la difficulté de reproduction des résultats. Dans le but de s'assurer d'une oxydation complète, Burn et Field (12) conseillent d'ajouter un oxydant au milieu réactionnel, alors que Townsend (13) recommande de saturer d'oxygène les solutions de départ.

Dans le mode opératoire suggéré au cours du présent travail, la réaction d'oxydation s'effectuant à une température plus basse (21° au lieu de 50° C), la solubilité de l'oxygène s'en trouve d'autant accrue.[2] De plus, la présence de molybdate d'ammonium favoriserait l'oxydation par l'oxygène qui est vraisemblablement une réaction relativement lente si l'on se rappelle qu'avec le réactif de Weil-Malherbe seul, on peut obtenir une fluorescence plus élevée, à la condition de prolonger la période de réaction à froid avant de porter le mélange à 50° C.

On sait déjà que l'oxydation de l'adrénaline est influencée par plusieurs ions, en particulier ceux des sels de métaux lourds, même à l'état de traces (14, 15). C'est précisément en étudiant l'action catalytique de divers ions métalliques (tels que Cu^{++}, Hg^{++}, Fe^{+++}, tungstates et molybdates) que nous avons reconnu l'avantage d'utiliser le molybdate d'ammonium. On sait aussi, depuis Cohen (16), qu'en milieu acide l'ion molybdate s'unit à l'adrénaline pour donner un complexe cristallisable. Il est plausible de supposer qu'une fois le milieu alcalinisé par l'éthylènediamine, ce complexe se décompose en une forme d'adrénaline à un niveau supérieur d'oxydation. Cette décomposition du complexe en milieu alcalin n'étant plus sujette à l'action catalytique des ions métalliques, la reproduction des résultats en est d'autant favorisée.

Résumé

Une modification de la méthode de Weil-Malherbe et Bone pour le dosage de l'adrénaline et de la noradrénaline est proposée. Elle consiste à faire réagir d'abord les catécholamines à la température ambiante avec le molybdate d'ammonium, puis les complexes molybdiques avec l'éthylènediamine en deux temps: à la température ambiante, puis à 50° C. Cette façon de procéder présente plusieurs avantages. Elle favorise une oxydation plus adéquate des amines (l'étape la plus cruciale de la réaction) et annule l'effet variable des impuretés métalliques, soit présentes dans les échantillons biologiques, soit accaparées lors de la séparation par adsorption sur alumine. De plus, elle augmente sensiblement le niveau de fluorescence, bien qu'elle exige moins d'éthylènediamine, diminuant ainsi le blanc des réactifs.

Contrairement à la méthode originale, le dosage différentiel des deux catécholamines s'effectue à une seule longueur d'onde grâce aux vitesses différentes de réaction des complexes molybdiques avec l'éthylènediamine.

[2]Dans la modification proposée, les solutions contiennent normalement assez d'oxygène dissous. Il est recommandable cependant d'agiter à l'air pendant quelques minutes les solutions étalons préparées à partir d'eau fraichement distillée.

Remerciements

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Bibliographie

1. Weil-Malherbe, H. et Bone, A. D. Biochem. J. **51**, 311 (1952).
2. Weil-Malherbe, H. et Bone, A. D. Lancet, **264**, 974 (1953).
3. Lund, A. Acta Pharmacol. Toxicol. **5**, 231 (1949).
4. Joly, L. P., Sobolewski, G. et Nadeau, G. Clin. Chem. **4**, 286 (1958).
5. Valk, A. de T. et Price, H. L. J. Clin. Invest. **35**, 837 (1956).
6. Mangan, G. F. et Mason, J. W. Science, **126**, 562 (1957).
7. Nadeau, G., Joly, L. P. et Sobolewski, G. Nature, **181**, 1061 (1958).
8. Mangan, G. F. et Mason, J. W. J. Lab. Clin. Med. **51**, 484 (1958).
9. Manger, W. M., Baldes, E. J., Flock, E. V., Bollman, J. L., Barkson, J. et Jacobs, M. Proc. Staff Meetings Mayo Clinic, **28**, 526 (1953).
10. Nadeau, G. et Joly, L. P. Can. J. Biochem. and Physiol. **37**, 231 (1959).
11. Erne, K. et Canbäck, T. J. Pharm. and Pharmacol. **7**, 248 (1955).
12. Burn, G. P. et Field, E. O. Nature, **178**, 542 (1956).
13. Townsend, E. Thèse, Université McGill, Montréal. 1956.
14. Chaix, P., Chauvet, J. et Jezequel, J. Biochim. et Biophys. Acta, **4**, 471 (1950).
15. Chaix, P., Morin, G. A. et Jezequel, J. Biochim. et Biophys. Acta, **5**, 472 (1950).
16. Cohen, G. N. Bull. soc. chim. biol. **27**, 237 (1945).

ISOLATION AND IDENTIFICATION OF THE FAST-DEATH FACTOR IN MICROCYSTIS AERUGINOSA NRC-1[1]

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Abstract

The fast-death factor in *Microcystis aeruginosa* NRC-1 is an acidic, probably cyclic peptide containing the following amino acids in the molar ratios indicated: L-aspartic (1); L-glutamic (2); D-serine (1); L-valine (1); L-ornithine (1); L-alanine (2); L-leucine (2). It is possible, although not likely, that one of the residues of glutamic, alanine, or leucine also is in the D-configuration. The toxin, in the form of its sodium salt, was extracted from lyophilized algal cells by water, separated from pigments by extraction into *n*-butanol, and freed from high-molecular-weight impurities by dialysis. No separation of a single toxic compound could be obtained by countercurrent distribution, chromatography, or electrophoresis in carbonate, acetate, or phosphate buffers. Electrophoresis of the crude toxin on cellulose in 0.1 *M* borate yielded five peptides one of which was toxic and accounted for 100% of the toxicity present in the crude preparation. The intraperitoneal LD_{50} of the pure toxin for mice was 0.466 ± 0.013 mg/kg body weight.

Introduction

There have been numerous reports of suspected poisoning by fresh-water algae from many parts of the world during the last 80 years. However, reports of attempts to isolate and characterize the toxins from poisonous algal growths are much less numerous. These are limited to six publications (1–6) and all are concerned with waterblooms in which species of the blue-green alga, *Microcystis*, were dominant. The work reported in these papers covers mainly the extraction of the toxin from the algal cells together with tests made either on the toxic water in which the algae were growing or on extracts prepared from the cells. Most of these tests were of a general nature such as for stability under various conditions, volatility, diffusibility, and solubility but others were more specific. Microchemical tests for various classes of compounds, such as alkaloids, botulinus, carbohydrates, amines, proteins, cyanide, and others predominated. The general tests were useful in defining some of the properties of the toxin and indicating possible methods for separating it. The reliability of the more specific tests must be regarded as questionable, however, since they were carried out on mixtures obtained by extraction of the algal cells. Many toxic compounds could be present in such mixtures in quantities below the level of detection. A summary of both the general and specific tests is given in Table I.

In all of the work cited, with the possible exception of that by Olson (6), the source materials were naturally occurring waterblooms. This dependence

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TABLE I

Properties of toxins associated with *M. aeruginosa* waterblooms

Reference	Solubility	Diffusibility	Stability	Negative tests	Other properties
Fitch *et al.* (1)	(s) Water	Passes through collodion or cellophane	Unstable to heat (100° C) when wet. Stable when dry	Alkaloid, toxalbumen, botulinus toxin	Non-volatile
Wheeler *et al.* (2)	(s) Water, alcohol (is) Ether, benzene, chloroform	Passes through collodion	Heat stable (autoclave) in neutral solution. Destroyed by heat in 1% alkali or acid. Gradual loss in unsterile solutions	Cyanide, amygdalin, acetylcholine; inconclusive tests for carbohydrate, amine, protein	Adsorbed on carbon—not eluted readily
Louw (3)	(s) Acetone, alcohol	———	———	———	Believed to be a toxic alkaloid
Shelubsky (4)	(s) Water, 95% ethanol, methanol (is) Chloroform ether, acetone, abs. ethanol	Passes through cellophane	Heat stable, alkali labile	Antigen or hapten. *Prymnesium* toxin	Adsorbed on carbon—difficult to remove. Not inactivated by human esterases. Superficially resembles *Gonyaulax* toxin
Stewart *et al.* (5)	(s) Water, methanol, ethanol, partially in acetone	Passes through Seitz filter	Stable to autoclaving for 2 hours, 90% loss when boiled to dryness	Metals, cyanide, protein, carbohydrate, alkaloid	Adsorbed on charcoal, not on dialyzed iron
Olson (6)	(s) Water, alcohol, acetone (is) Benzene, ether, chloroform	Passes through cellophane and animal membranes	Relatively heat stable	Cyanide, nitrate, nitrite, pilocarpine, saponins, cardiac glycosides, alkaloids	Non-volatile, resistant to extreme changes in pH. Appears to be more than one substance involved

on naturally occurring sources imposes severe limitations on the amount of material available for study and the variability of toxicity, number of algal species present, and degree of bacterial contamination make interpretation of results highly uncertain.

Olson (6) was the first to isolate and culture a unialgal strain of *M. aeruginosa* which was found to be toxic. His strain apparently had very low toxicity and he concluded that the very large quantities of algae required for the study of the toxin could be obtained most economically from naturally occurring blooms.

More recently, however, Hughes, Gorham, and Zehnder (7) have reported the isolation and unialgal culture of another strain of *M. aeruginosa*, designated NRC-1, which is much more toxic. An endotoxin produced by this strain causes fast deaths when injected intraperitoneally into white mice. The accompanying symptoms are similar to those which have been described for a number of toxic waterblooms. The concentration of this fast-death factor (FDF) varies with the developmental stage of a culture, and at maximum can equal that of a very toxic waterbloom. Since they were able to grow this strain in good yield, they suggested that it was feasible to abandon the collection of blooms and to grow large quantities of *M. aeruginosa* NRC-1 for the isolation and identification of the toxic factors instead. In addition to the FDF, Hughes *et al.* (7) detected the variable presence of a slow-death factor (SDF) in their cultures. They suggested that the source of SDF might be bacterial contaminants. Work from these laboratories, to be reported elsewhere, confirms this view and in addition indicates that FDF is algal in origin. This paper will be concerned only with the toxin which has been called FDF, obtained from mass cultures of *M. aeruginosa* NRC-1, and will describe the steps leading to its identification.

Experimental

Bio-assay of Toxin

Throughout this work white mice were used as test animals to detect and assay toxicity. The mice varied in weight between 15 and 25 g and testing was carried out by intraperitoneal injection of aqueous solutions or suspensions. A unit of toxicity termed a "mouse unit" (M.U.) was established and used to assay for recoveries or loss of toxin. This unit was defined as the minimum amount of toxin required to kill a 20-g mouse. To assay the toxin in various fractions the following procedure was adopted: An aliquot was diluted with water until 0.1 ml contained approximately 1 M.U., assuming 100% recovery of toxicity. The diluted solution was then injected into mice at doses of 0.05, 0.10, 0.20, 0.40, and 1.0 ml and the titer determined. Two mice per dose were used except for the determination of the LD_{50} of the pure toxin when 10 mice per dose were used. Sterilized syringes and needles were used throughout but samples were prepared with unsterilized water in clean, unsterilized glassware.

Mass Culture and Harvest of M. aeruginosa NRC-1

The mass culture of *M. aeruginosa* NRC-1 was carried out at 25 to 28° C in unsterilized culture solution No. 11 (7) prepared with deionized tap water. Plain, round, 9-l. Pyrex serum bottles were used for containers. Each 8-l. culture was inoculated with approximately 250 ml of aseptically grown inoculum containing 0.2 to 0.4 g dry weight of cells. The cultures were placed on either side of three long rows of 40-w "White" fluorescent lamps spaced one above the other about 6 in. apart. The light intensity (*ca.* 500 ft-c) was adequate for optimal growth throughout all but the most terminal stages of development. Each culture was aerated and stirred by filtered, compressed air which was passed through a vertical type, coarse, fritted disk, gas-dispersing tube at a rate in excess of 2 l. per min. There were facilities for growing nearly 300 cultures of varying ages at one time. The gas-dispersing tubes were cleaned and autoclaved after each use; the bottles were cleaned and autoclaved after being used four or five times.

The inoculum was subcultured aseptically in solution No. 11 in two stages. Three 3-l. shake-flasks, each containing 0.9 l. of solution plus 0.1-l. inoculum from a fully grown culture, were incubated at 160 ft-c, 28 ± 0.1° C, and 500 cc/min aeration (7). The harvests from the first stage were used to supply inoculum for the second stage which consisted of four 8-l. cultures, as described above, except that the bottles were filled, the tops protected by aluminum foil, and autoclaved before use.

Daily harvests were made of 25 to 50 cultures that had been growing for 4 or 5 days and were judged by color to have reached the point of maximum toxin production (7). These were replaced by freshly inoculated cultures. The harvested cultures were poured into 15-gal polypropylene settling tanks and aluminum sulphate was added to coagulate the cells (7). After the coagulum had settled, the supernatant was siphoned off and discarded and the remaining cell concentrate was centrifuged in 1-l. polyethylene cups at $1350 \times g$ for 5 minutes. The cells were transferred with a minimum of water to large trays, frozen at −12.5° C, and lyophilized. The lyophilized cells were passed through a 40-mesh sieve, bottled, and stored at room temperature. The maximum rate of production was about 1.0 kg dried cells per month, having an LD_{100} (7) varying between 80 and 160 mg/kg body weight.

Extraction of Toxin

Earlier reports (1–6) described some of the general properties of algal toxins (summarized in Table I) and were useful here in indicating possible methods for extracting the toxin from the high-molecular-weight polysaccharides and proteins of the cell wall. The properties shown in Table I indicated that extraction with methanol or 95% ethanol should remove the toxin from the cell wall material and that it could be further purified by dialysis.

Procedure (a)

The freeze-dried algae cells were extracted in a Soxhlet apparatus with methanol or 95% ethanol. Extractions were exhaustive, being continued

with fresh changes of solvent, until no more toxin was removed. Bio-assay of the extracts and residual cells showed that 80–85% of the toxin was removed by alcohol extraction. The alcohol extract was then evaporated to a heavy sirup which was suspended in water by vigorous shaking and dialyzed through Visking tubing against distilled water at 5° C. The dialyzate was removed every 24 hours, concentrated, and tested for toxicity. After 4 days, 50% of the total toxicity had been recovered in the dialyzate and this increased to 80% after 8 days. The remaining toxicity was found in the non-dialyzables. Evaporation of the dialyzate yielded a yellow-brown sirup which was not completely soluble in water but dissolved readily in 50% aqueous ethanol. A stock solution of the dialyzable material in 50% aqueous ethanol was found to retain its toxicity indefinitely when stored at 5° C and was used for preliminary experiments on the properties of the toxin. The LD_{100} of the solids in the dialyzate was of the order of 8 mg/kg body weight.

This procedure was not entirely satisfactory because it left 15–20% of the toxin in the residual cells. Furthermore, a large portion of the material in the extract was insoluble in water forming a gummy precipitate which was difficult to remove by filtration or centrifugation and which plugged the dialysis membrane. However, sufficient material was obtained by this procedure for some preliminary experiments during which it was found that the toxin was acidic and that its solubility properties depended on whether it was present as the free acid or as a salt. This discovery led to the improved extraction procedure described below in which the toxin was converted into its sodium salt which was water-soluble and could be extracted at room temperature.

Procedure (b)

Freeze-dried cells (100 g) were mixed thoroughly with 0.1 *N* sodium carbonate (850 ml) in two stages, first with 250 ml then with 600 ml. The mixture was centrifuged in a polyethylene bottle at $750 \times g$ for 30 minutes and the supernatant was decanted. The residue was extracted in the same way with 0.1 *N* sodium carbonate (600 ml) and then with three lots of 0.02 *N* sodium bicarbonate (600 ml each). It was necessary to keep the pH above 7 to assure conversion to the sodium salt, but below 10.0 to avoid losses by alkali degradation (see section on stability). The combined extracts were passed through a Sharples centrifuge and emerged as a bright-blue solution. This solution was concentrated at 35° C under reduced pressure to about 1/10 its volume and extracted with *n*-butanol. Centrifugation was required to separate the phases as the blue phycocyanin pigments separated out at this stage. The combined butanol extracts were evaporated to dryness under reduced pressure and the residue was dissolved in water. This solution was washed with ethyl acetate to remove some colored material and the aqueous phase, containing the toxin, was dialyzed through Visking tubing against distilled water at 4° C using stirring. After 40 hours, over 60% of the toxin had dialyzed through the membrane. Further dialysis for 90 hours against

a fresh change of water removed another 15% of the toxin. Prolonged dialysis eventually removed all of the toxin from the non-dialyzable material. Because the aqueous dialyzate tended to foam badly the toxin was recovered by extraction into *n*-butanol and stored at 5° C as its sodium salt either in ethanol or water. No detectable toxicity was left in the residue after the carbonate–bicarbonate extraction. Total recovery of toxicity was 80–85%.

Two stock solutions were made up from the combined dialyzates of a number of extractions carried out as described above. *Solution* (*a*): the dialyzables (2 g on a dry weight basis) in the free acid form were made up to 50 ml with 95% ethanol. Bio-assay showed that this solution contained 50,000 M.U. On a dry weight basis the LD_{100} of the toxin in this form was 2.0 mg/kg. *Solution* (*b*): the dialyzables (2 g on a dry weight basis) in the sodium salt form were made up to 100 ml with water. Bio-assay showed that this solution also contained 50,000 M.U. and again the LD_{100} was 2.0 mg/kg. These two solutions, (*a*) and (*b*), were used as the source of toxin for all subsequent work.

Mice injected with solution (*a*) or (*b*) showed the same symptoms of toxicity as those injected with aqueous suspensions of lyophilized cells prior to extraction. This was taken as evidence that the toxin had not been altered by the extraction procedure.

Test of Volatility

An unsuccessful attempt was made to purify the toxin by distillation or sublimation *in vacuo*. From the solids in solution (*a*) small amounts of non-toxic distillates were recovered at 100° C/0.03 mm Hg and at 195 to 200° C/0.03 mm Hg. The amount of toxin used (500 M.U.) would have assured easy detection had any of it distilled. Fitch *et al.* (1) and Olson (6) also found that the algal toxin was non-volatile.

Behavior Toward Ion Exchange Resins

Solution (*b*) (0.2 ml, 4 mg, 100 M.U.) was washed through a column containing 21 ml of a basic exchange resin, Amberlite IR-4B (OH form). The column was washed with water until the eluate was colorless. The eluate was concentrated to 10 ml and bio-assay of this solution showed complete recovery of the toxin. The experiment was repeated in exactly the same way using an acidic exchange resin, Amberlite IR-120. No toxin was recovered. This seemed to indicate that the toxin was a base but when its acidic character became clear it was obvious that the acidic resin was removing the cation from the salt form of the toxin causing precipitation of the water-insoluble acid form in the column.

Solubility Properties

Two procedures were used to test solubility: The first was for solvents miscible with water and the second for solvents immiscible with water. (1) Samples of solution (*a*) (0.25 ml, 10 mg, 250 M.U.) were evaporated to dryness and the residues were shaken with the solvents to be tested. The solvent

was then decanted, evaporated, and any material that had been extracted was dissolved in about 1 ml of ethanol, diluted to 25 ml with water, and tested for toxicity. (2) Samples of solution (*b*) (0.5 ml, 10 mg, 250 M.U.) were diluted with water (2.5 ml) and extracted continuously with the solvent being tested. After 24 hours the extract was removed, the aqueous phase was acidified (pH 2) with hydrochloric acid, and the extraction was repeated. Extracts from both the neutral and acidic solutions were evaporated separately, dissolved in ethanol (1 ml), diluted to 25 ml with water, and tested for toxicity.

The toxin was found to be soluble in methanol, ethanol, *n*-butanol and, as its sodium salt, in water. It was insoluble in ether, petroleum ether, benzene, chloroform, carbon tetrachloride, ethyl acetate, carbon disulphide and, as the free acid, in water. Low solubility of the toxin was observed in acetone and 2-butanone. These solubility properties are in good agreement with those previously reported (cf. Table I) all of which were based on the extractability of the toxin from the algal cells by the solvent being tested. Any differences in solubility in water or alcohol become clear when it is considered that the toxin undoubtedly is present in the algae in both the free acid and salt forms.

Stability Tests

The toxin was tested for its stability towards different solvents and towards aqueous solutions at various pH levels. To check for any losses caused by photochemical reaction these tests were run in duplicate, one under a fluorescent lamp, the other in the dark. Samples of solution (*b*) (0.5 ml, 250 M.U.) were diluted to 10 ml with water, 10 ml of the solvent or buffer to be tested was added, and the solutions were shaken continuously. The following solvents and buffers were tested: water, methanol, ethanol, ethyl acetate, 2-butanone, 2-butanone–ether (8:2), and acetic acid – sodium acetate – sodium hydroxide buffer at pH levels of 1.0, 7.0, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, 11.0, 12.0. The solvent-treated samples were tested for toxicity at 24-hour intervals for 6 days. Solvents were removed by evaporation, the residues were dissolved in water, and bio-assayed. The buffer-treated samples were tested after 3, 8, 24, and 50 hours and were prepared for bio-assay simply by adjusting the pH to approximately 7 with 0.1 *N* hydrochloric acid or 0.1 *N* sodium carbonate. The results showed that the toxin was stable towards all solvents tested except 2-butanone in which 95% of the toxicity was lost after 5 days. The toxin was stable in all buffers up to and including that at pH 10.0. In agreement with Wheeler *et al.* (2) and Shelubsky (4) it was found that toxicity was destroyed at higher pH levels. There was no loss after 3 or 8 hours, 25–50% loss after 24 hours, and 75–100% loss after 50 hours. There were no differences between the samples exposed to light and those kept in the dark.

No specific tests were made for stability to heat or long periods of storage. Evaporations were carried out routinely at 40° C and materials were stored at 5° C for periods up to 1 year. No losses occurred under these conditions.

Tests for Antibiotic Activity

Solutions (*a*) and (*b*) at concentrations of 4.3 mg/ml and 1.0 mg/ml respectively were tested for antibiotic activity against two strains of *Bacillus subtilis* and one strain each of *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas hydrophila*. The cut plate method of assay was used; no inhibition was observed. The two strains of *B. subtilis* and the one strain of *S. aureus* were sensitive to penicillin and aureomycin.

Countercurrent Distribution Studies

For these studies, quantities of the dialyzate from extraction procedure (*a*) containing 2000 to 4000 M.U. were used. Partition coefficients in various solvents were determined and are listed in Table II. These values were determined by shaking the appropriate volume of dialyzate with an equal

TABLE II

Partition coefficients of toxin in various solvents

Solvent	Partition coefficient
Amyl acetate:water	<0.01
Ethyl acetate:water	<0.05
Amyl alcohol:water	14.0
Ethyl acetate (1):butanol (1): water	6.0
Ethyl acetate (2):butanol (1): water	1.5
Ethyl acetate (4):butanol (1): water	0.2
2-Butanone:water	1.0

volume of the water-saturated organic phase. The two phases were then separated, solvent was removed by evaporation and the residues were redissolved in water for bio-assay. It can be seen that with the esters, amyl acetate and ethyl acetate, the toxin remained almost completely in the aqueous phase. On the other hand, with the alcohols, amyl alcohol and *n*-butanol most of the toxin was found in the organic phase. Addition of ethyl acetate to the *n*-butanol decreased the partition coefficient of the toxin in this solvent. Thus the system ethyl acetate:*n*-butanol:water (2:1:saturation) gave a nearly ideal value of 1.5. The system 2-butanone–water gave an ideal partition coefficient of 1.0 but could not be used because of instability of the toxin in 2-butanone.

A portion of the dialyzate from procedure (*a*) (2000 M.U.) was distributed in ethyl acetate:*n*-butanol:water (2:1:saturation) using a hand-operated countercurrent distribution apparatus. After 40 transfers the contents of each tube were evaporated leaving residues which were dissolved in water for bio-assay. Two peaks of toxicity were found, one (A) having its maximum at tube 21, the other (B) at tube 38. The distribution curve is shown in Fig. 1. Total recovery of toxicity was 1700 M.U., 85%. Evaporation of the contents of tubes 17–24 yielded component A which had an LD_{100} of 3 mg/kg; tubes 35–40 yielded component B, which had an LD_{100} of 2 mg/kg.

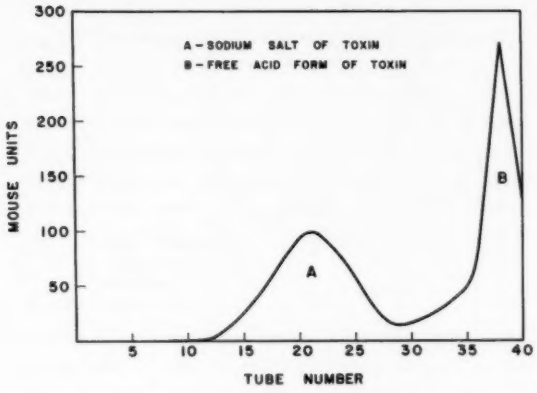

FIG. 1. Countercurrent distribution of toxic dialyzate.

This experiment revealed the acidic nature of the toxin. It was found that the solubility properties of the two components could be reversed by acidification or neutralization according to the following system.

$$\text{Component A} \underset{CO_3^-}{\overset{H^+}{\rightleftharpoons}} \text{component B}$$

The countercurrent distribution was therefore separating only the salt form (component A) from the free-acid form (component B) of the toxin, both of which were extracted from the algae cells by 95% ethanol. These results led to the improved extraction procedure (*b*) described earlier. Components A and B each had the same LD_{100} (2–3 mg/kg) as the material in the dialyzate from procedure (*b*) showing that no purification of the toxin was being obtained by countercurrent distribution. In addition to this the partitioning system formed extremely stable emulsions which made it impossible to use an automatic apparatus or a larger number of transfers. Addition of enough solvent, e.g. ether, to break these emulsions caused a large alteration in the partition coefficient. For these reasons no further attempts were made to purify the toxin by countercurrent distribution.

Chromatography

(a) Paper Chromatography

Preliminary chromatograms were run on 13×50 cm strips of Whatman No. 1 paper using a descending flow of *n*-butanol saturated with water. The dialyzate was applied to the paper in the form of a band and an amount was used equivalent to 100 M.U. Attempts were made to find a spray reagent that would give a positive reaction in the area of the chromatogram which was found to contain toxicity. The following reagents were tried: (*a*) silver nitrate and alkali, (*b*) alkaline potassium permanganate, (*c*) ninhydrin, (*d*) resorcinol in hydrochloric acid, (*e*) methyl orange – Congo red,

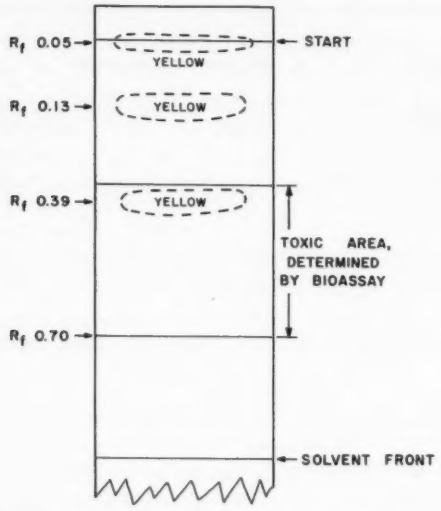

FIG. 2. Chromatogram of toxic dialyzate. Solvent: *n*-butanol saturated with water.

(*f*) iodine vapor, and (*g*) Draggendorff's reagent. Negative results were obtained with all of these reagents. A typical chromatogram is shown in Fig. 2. The three visible yellow bands had R_f values of 0.39, 0.13, and 0.05. Each of the three yellow bands and the blank areas between them were sectioned out and eluted separately with 90% aqueous methanol. The eluates were evaporated, taken up in water, and tested for toxicity. The toxin was found in the area from R_f 0.39 to R_f 0.70 and included the fastest-moving yellow band. Because part of the toxin was separated from this yellow band it appeared that the two were overlapping in this solvent system. Chromatograms were run in *n*-butanol:*n*-propanol:water (3:1:saturation) in an attempt to separate the toxin from the pigments. This solvent increased the R_f values of the yellow pigments to 0.10, 0.20, and 0.50; bio-assay of sections of the paper as before showed that the toxin was contained in an area extending from R_f 0.63 to R_f 0.87 and was completely separated from the yellow pigments. A number of chromatograms were run in this solvent and the toxic areas were combined and eluted. Recovery of toxicity was low (50%) and the LD_{100} of 7 mg/kg showed that it was grossly contaminated with material coextracted from the paper. No further attempts were made to use paper chromatography as a preparative method for isolating the toxin. However, the discovery that the toxin gave a strong positive reaction with a chlorine:potassium iodide:tolidine reagent (8) raised the possibility that paper chromatography could be used to follow separations and perhaps as an indication of purity. Chromatograms run in *n*-butanol:*n*-propanol:water (3:1:saturation) were chlorinated and then dipped in a potassium iodide – tolidine solution (8). An intense blue color appeared in the area

R_f 0.63 to R_f 0.87 coinciding with the toxin and trailing off back to the starting line. No single discreet spot could be detected but there appeared to be several overlapping spots of intense coloration. A variety of other solvent systems were tried including some which contained acid or alkali. No improvement in the separations was obtained. Severe trailing was encountered with all solvents probably because of adsorption on the surface of cellulose. This adsorption of the toxin by cellulose was evident from the R_f values, which were much lower than would be expected from the partition coefficients in similar solvents and undoubtedly accounted for the low recovery.

(b) Column Chromatography

Attempts were made to isolate the toxin from the dialyzate from extraction procedure (*a*) by partition chromatography on columns of cellulose or acid-washed Celite 535 using *n*-butanol saturated with water. Recovery of toxin was 50% from the cellulose column and less than 15% from the Celite column. The toxicity recovered from both of these chromatograms was contained in a large volume of effluent which indicated severe trailing. An attempt to isolate the toxin by chromatography on charcoal resulted in complete and irreversible adsorption of the toxin by the charcoal (cf. Table I, references 2, 4, and 5).

Behavior of Proteolytic Enzymes Towards Toxin

Aliquots of solution (*b*) (1 ml, 500 M.U.) were mixed with 2 mg of the enzyme preparations and the mixtures were diluted to 20 ml with a buffer that gave the optimum pH conditions for the enzyme used. The solutions were then allowed to stand in the dark at room temperature (25–30° C). At intervals of 24, 36, 48, 96, and 120 hours samples were withdrawn for bio-assay and chromatography. The following enzymes were used: pepsin, papain, trypsin, chymotrypsin, carboxypeptidase, polidase, takadiastase, and takadiastase: papain. None of the enzymes caused any loss of toxicity nor could any ninhydrin-positive material be found on chromatographic examination of the digests.

Partial Acid Hydrolysis

Solution (*a*) (1 ml, 1000 M.U.) was evaporated to dryness and the residue was dissolved in glacial acetic acid:10 *N* hydrochloric acid (1:1, 2 ml). This solution was then stored at 37° C and samples were removed for bio-assay and chromatography after 24, 48, 70, and 94 hours. For bio-assay 0.06 ml of the solution was diluted to 3 ml and for chromatography 0.05 ml of the solution was spotted on the paper. The hydrolysis caused a loss of 50% of the toxicity in 24 hours, 75% in 48 hours, and 100% in 70 hours. Chromatography revealed the presence of free amino acids in the hydrolyzate at all intervals. The amounts increased with time (or with decreasing toxicity). Chromatograms of these amino acids were identical with that obtained from a total hydrolyzate of the material in solution (*a*). This latter hydrolysis was accomplished by heating the material with 10 *N* hydrochloric acid in a sealed tube at 105° C for 20 hours.

Electrophoresis

The behavior of the toxin when subjected to paper electrophoresis was studied. For all electrophoresis experiments the following procedure was used: solution (*a*) (0.3 ml, 300 M.U.) was streaked as a band across the middle of a strip (6.5×46.5 cm) of Whatman 3MM paper. The strip was then dipped in buffer and subjected to electrophoresis in a "sandwich" type of apparatus (9). At the end of electrophoresis the strip was dried and cut in half, lengthwise. One half was then sectioned crosswise into 1-in. segments which were eluted with water, the eluates then being bio-assayed. The other half of the paper electrophorogram was developed with the chlorine: potassium iodide:tolidine reagent. In the early experiments electrophoreses were carried out at 1000 v and 5–20 ma (dependent on buffer) for 1 to 6 hours. At pH values of 2.2 to 5.0 (acetic acid or acetic acid:ammonium acetate buffers) the toxin behaved as a non-ionic substance. The area containing the toxin corresponded with that giving a positive reaction with the chlorine: potassium iodide:tolidine reagent. However, this band was intense in the middle and diffuse on both sides and no sharp separation was obtained. At pH values higher than 7.0, e.g. 9.2 (sodium carbonate:sodium bicarbonate buffer) the toxin moved toward the anode as a streak. The indicating reagent also showed a streak moving towards the anode and occurring in the same areas that contained the toxin. This streak contained several diffused spots within the toxic area as well as some other faster-moving components and some non-migrating components. A typical electrophoresis of this type is shown in Fig. 3. This result was obtained in carbonate:bicarbonate buffer

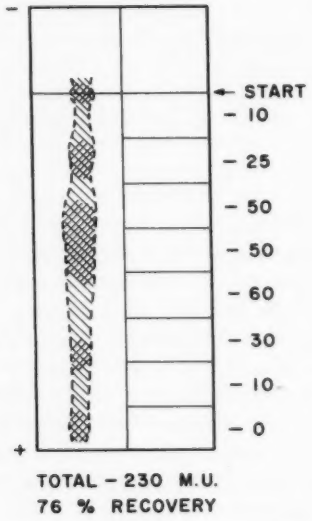

FIG. 3. Electrophoresis of toxic dialyzate in acetate: Numbers refer to mouse units recovered by elution. Indicator was chlorine:potassium iodide:tolidine.

(0.02 *N* Na^+) at pH 9.2 by electrophoresis at 1000 v and 10 ma for 2½ hours. Similar results were obtained with phosphate and acetate buffers at the same pH. Buffers at higher pH values could not be used because of the instability of the toxin above pH 10.2. Because of the possibility of adsorption on the cellulose surface being responsible for the streaking, electrophoreses were also run using glass fiber sheets (10) and sponge rubber (11) as supports. No improvement in the separation was obtained.

It was found that on electrophoresis in 0.1 *M* borate buffer (pH 9.1) on glass fiber sheets the toxin moved as a single discreet band. The toxicity corresponded exactly with the material which gave a positive peptide reaction with the chlorine:potassium iodide:tolidine reagent. When the support was changed from glass fiber sheets to Whatman 3MM paper, electrophoresis at 650 v and 25 ma for 6 hours gave a complete resolution of five components (Fig. 4).

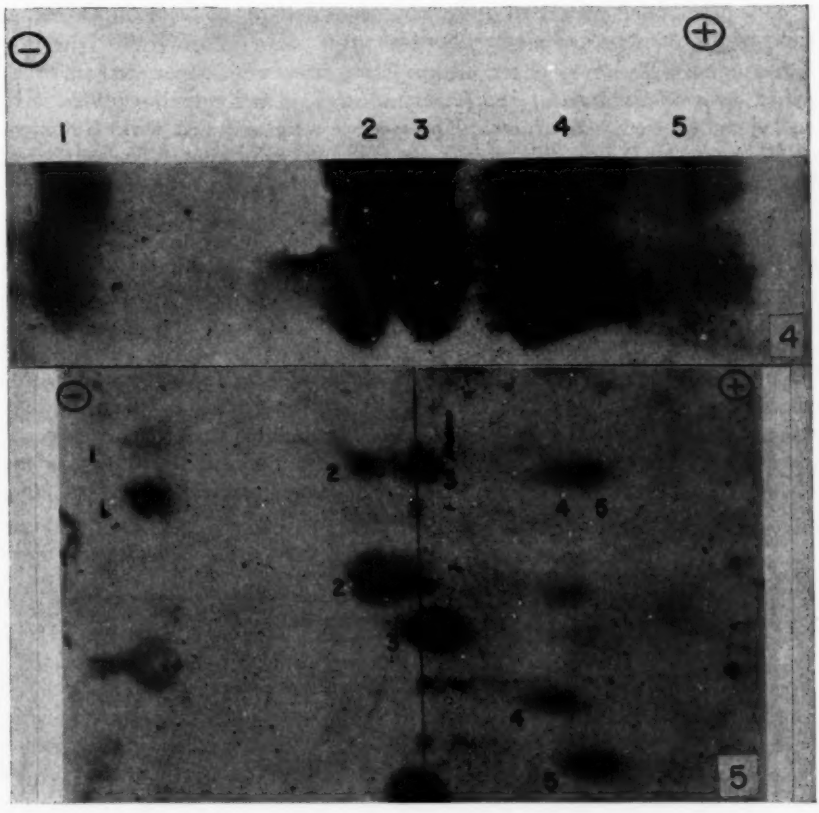

FIG. 4. Electrophoresis of toxic dialyzate in borate buffer.
FIG. 5. Electrophoresis of separated peptides in borate buffer.

Solution (*b*) (0.5 ml, 250 M.U.) was applied as a band (15 cm long) on the starting line located halfway along a strip of Whatman 3MM paper (20× 46.5 cm). The paper was dipped in 0.1 *M* borate buffer (pH 9.1) and subjected to electrophoresis at 650 v and 100 ma for 6 hours. The paper was dried and guide strips (1 cm wide) were cut from the middle and both edges. These strips were developed with the chlorine:potassium iodide:tolidine reagent and were then used to locate the separated peptides on the undeveloped strips. The separated peptides were eluted from the paper with water and the eluates were bio-assayed.

Only peptide No. 2 (Fig. 4) was toxic. The blank areas of the paper were also tested and yielded no toxicity. Toxin recovered was equivalent to 200 M.U. and since 3 cm from the 15-cm band (or 20%) had been sacrificed to locate the peptides this represented a recovery of 100%. The separated peptides were subjected to electrophoresis again using the original mixture as a control standard. The result (Fig. 5) showed that the peptides were electrophoretically pure and were not separated or converted into other components on repeated electrophoresis.

A number (20–30) of paper electrophorograms were run exactly as described above. Sections of the papers containing individual peptides were pooled and eluted with water. The eluates were acidified with *N* hydrochloric acid and the peptides were extracted into *n*-butanol. The *n*-butanol was then evaporated under reduced pressure at 40° C and the residues were concentrated 10 times from methanol to assure complete removal of borate. The solutions were then evaporated to dryness and the residues were dried at 0.03 mm Hg over anhydrous calcium chloride to yield peptides 1, 2, 3, 4, and 5 in quantities of 5.3, 9.0, 8.4, 6.9, and 8.9 mg respectively.

Amino Acid Composition of Peptides

Samples (2–3 mg) of each of the five peptides were heated with 10 *N* hydrochloric acid (0.5 ml) in sealed tubes at 105° C for 20 hours. The hydrolyzates were spotted on the starting point of a two-dimensional paper chromatogram. Excess hydrochloric acid was removed by aeration of the paper for 24 hours. The chromatograms were developed in one direction with 80%

TABLE III

Amino acids present in five peptides from *M. aeruginosa*

Amino acids	Peptide				
	1	2	3	4	5
Aspartic	+	+	+	+	+
Glutamic	+	+	+	+	+
Serine	+	+	+	+	+
Valine	+	+	+	+	+
Ornithine	+	+	+	+	+
Alanine	−	+	+	+	+
Leucine	−	+	−	−	−
No. of unknowns	0	0	4	4	3

phenol, dried, and then developed twice in the other direction with *n*-propanol:ethyl acetate:water (7:1:2) (12). All developments were by the ascending method. To locate the amino acids the dried chromatograms were sprayed with ninhydrin in 95% ethanol buffered to pH 7.0 and heated at 95° C. Table III shows the amino acids found in the five peptides. Further chromatographic identification of these amino acids was obtained by unidimensional chromatography of the hydrolyzates in *n*-butanol:acetic acid:water (4:1:1) and by recovery of the individual amino acids from a unidimensional chromatogram and co-chromatography of them with an authentic sample in the two-dimensional system described above. Still further chromatographic identification was obtained by two-dimensional chromatography of the *N*-2,4-dinitrophenyl derivatives in *tert*-amyl alcohol and phosphate buffer (13, 14).

Quantitative Estimation of Amino Acids in Peptide 2

Amino acids in the hydrolyzate of peptide 2 were estimated spectrophotometrically as their *N*-2,4-dinitrophenyl derivatives (14). The *N*-2,4-dinitrophenyl derivatives were separated chromatographically as described above and the individual components were eluted from the paper. Measurement of the per cent transmittance of the eluates at 360 mμ by a Beckman spectrophotometer gave the following approximate molar ratios of amino acids:

Aspartic	— 1	Ornithine	— 1
Glutamic	— 2	Alanine	— 2
Serine	— 1	Leucine	— 2
Valine	— 1		

Determination of LD_{50} of Peptide 2

Another sample of the toxic peptide (13.0 mg), isolated as described above, was dissolved in ethanol and the solution was diluted to exactly 10 ml. Estimation of LD_{100} showed that this solution contained 2200 M.U. From this solution 0.227 ml (50 M.U.) was pipetted and diluted to 25 ml with water. This solution was then bio-assayed at 0.40, 0.45, 0.50, 0.55, 0.60, 0.65, and 0.70 ml, 10 mice being injected at each dosage level. Analysis of the resulting probit curve gave $LD_{50} = 0.593 \pm 0.016$ ml for a 15-g mouse. On a weight basis this gave $LD_{50} = 0.466 \pm 0.013$ mg/kg body weight.

Examination of Peptide 2 for D-*Amino Acids and* N-*Terminal Amino Acids*

The hydrolyzate of peptide 2 was chromatographed in *n*-butanol:acetic acid:water (4:1:1) by the descending method. The paper was dried overnight and was then sprayed with a solution of D-amino acid oxidase and incubated at 37° C in an atmosphere of moist oxygen for 2½ hours (15). The chromatogram was then sprayed with ninhydrin, heated, and compared with an identical chromatogram that had not been sprayed with the oxidase. The results showed that serine had been almost completely removed by the D-amino acid oxidase, the other amino acids remaining unchanged. It is

possible that one residue of each of the amino acids that occurred in bimolar proportions, glutamic, alanine, leucine, could have been in the D-configuration and escaped detection by this method. However, no decrease in the spot intensities of these amino acids was observed.

Peptide 2 (1.17 mg) was shaken with aqueous sodium bicarbonate and 5% ethanolic 2,4-dinitrofluorobenzene for 2 hours (16). The alkaline solution was extracted with ether to remove excess 2,4-dinitrofluorobenzene and the aqueous residue was evaporated to dryness. Hydrochloric acid (6 *N*) was then added and the solution was heated at 95° C for 60 hours. The solution was diluted to 1/5 its acid strength with water and extracted with ether to remove any *N*-2,4-dinitrophenyl amino acids. The extract was evaporated to dryness, 2,4-dinitrophenol was removed by sublimation, and the residue was chromatographed in two dimensions in *tert*-amyl alcohol and phosphate buffer. No *N*-2,4-dinitrophenyl amino acids could be found on the chromatogram.

Discussion

Although the initial experiments using countercurrent distribution, chromatography, and ion exchange resins were unsuccessful in providing any purification of the toxin beyond the dialysis step, they were important because of the information about the chemical nature of the toxin that could be deduced from them. They showed that the toxin was a non-volatile acid with a strong affinity for adsorptive surfaces. The reagents tested on chromatograms included those that would react with any reducible or oxidizable components, amino acids, alkaloids, and most nitrogen-containing compounds. All of these gave negative results indicating that perhaps the toxin was present in amounts below the level of detection by these reagents and that its potency was such that it could only be detected by bio-assay. However, a strong reaction with the chlorine:potassium iodide:tolidine reagent (8) in the area of greatest toxicity on chromatograms and electrophorograms provided a visible means of following the toxin through purification procedures and indicated that it was probably a peptide. Although this reagent will give a positive test with practically any nitrogenous compound having an $>$N—H or —NH_2 grouping it was possible to eliminate such compounds, other than peptides, by the solubility properties of the toxin and the negative tests obtained with the other reagents tried. For example, alkaloids should have given a positive test with iodine vapor or Draggendorff's reagent and glycosides most certainly would have reacted with alkaline silver nitrate or alkaline permanganate. Additional evidence of the peptide character of the toxin was obtained by partial hydrolysis of the mixture when amino acids were released in increasing amounts as the toxicity decreased. Although absorption spectra of mixtures are difficult to interpret, the ultraviolet and infrared spectra of the material in the dialyzate were consistent with the presence of peptides.

Fitch *et al.* (1) reported that the toxin did not migrate in an electric field but the conditions used were not described. In the present work the toxin behaved as a non-ionic substance on electrophoresis at pH values of 2.2 to 5.0 but moved to the anode at pH values above 7.0. To avoid destruction of the toxin by alkali it was, of course, necessary to keep the pH of the buffer below 10.0. It was finally found that a complete separation of five components, all giving a positive peptide reaction, could be obtained by electrophoresis in 0.1 *M* borate buffer at pH 9.1 (Fig. 4). These five components were isolated by preparative paper electrophoresis and only one of them, peptide 2, was toxic. Furthermore 100% of the toxin applied to the paper was recovered in this one component.

It was interesting that the five peptides were separated by electrophoresis in borate buffer and not in other buffers under exactly the same conditions. This result showed that the peptides must have formed complexes with the borate, possibly through their >N—H groups, which were then separated on the basis of charge differences. It was also found that the same separation could not be obtained using glass fiber paper when all five peptides moved together in one single band. The borate complexes then must have taken the form of cross links between the peptides and the cellulose, the difference in number of these cross linkages being responsible for the separation by electrophoresis. Cooper (17) has reported the separation of a new protein from bovine serum by electrophoresis in borate and attributed the result to reaction between borate and a protein-bound carbohydrate. This could not be the reason for the separations obtained in the work reported here. First of all, had there been carbohydrate in the mixture capable of complexing with borate then the separations should have been obtained on glass fiber paper as well as cellulose. Secondly, no carbohydrates could be found in acid hydrolyzates of the five peptides. It appears, therefore, that paper electrophoresis using borate buffer may prove generally useful as a method for separating peptides. From evidence discussed below, peptide 2 (the toxin) is probably cyclic and so, probably, are the other four. It may be that the ring forms were involved critically in the formation of the borate complexes but there is not sufficient data to clarify this point.

On acid hydrolysis the peptides yielded the amino acids listed in Table III. The five peptides were closely related in that the first five amino acids were common to each of them. It was also noted that the toxin (peptide 2) was the only peptide containing leucine. No further attempts were made to identify the unknowns in peptides 3, 4, and 5—the remainder of the work being concentrated on the toxin.

Identification and quantitative estimation of the amino acids in peptide 2 showed that the toxin was composed of 10 amino acid residues. Of course, depending on the molecular weight, the actual size of the toxin could be any multiple of this number but a low figure is favoured because of the rate at which the toxin passed through the dialysis membrane. A chromatographic method for detecting D-amino acids (15) showed that at least the serine in

peptide 2 was in the D-configuration. Application of Sanger's (16) method for identifying N-terminal amino acids showed that there was no N-terminal α-amino acid in peptide 2 and therefore that it was probably cyclic. Under the conditions used only α-amino N-terminal acids would have been detected. In other words, the δ-amino group of the ornithine in peptide 2 could be free but the α-amino groups must have been tied up. The presence of two dicarboxylic acids, aspartic and glutamic, in a cyclic peptide provide an explanation of its acidic character.

The assignment of a cyclic structure to peptide 2 was supported by the difficulty of hydrolysis by enzymes. Consden *et al.* (18) found that the cyclic peptide, gramicidin S, was unusually resistant to acid hydrolysis and attributed this property to the immobilization of the peptide bond from rotation in the relatively rigid cyclopeptide structure. Sanger (19) has added that "such an effect might also partly account for the relative stability of native proteins to enzymic hydrolysis". Peptide 2 showed no signs of hydrolysis, either by disappearance of toxicity or release of amino acids, when incubated with pepsin, papain, trypsin, chymotrypsin, carboxypeptidase, polidase, takadiastase, or takadiastase:papain. This resistance to enzymatic hydrolysis could be a significant factor in the oral effectiveness of toxic waterblooms.

It is not within the scope of this paper to discuss in any detail the relation between the toxic peptide from *M. aeruginosa* NRC-1 and other toxins or other naturally occurring peptides. The symptoms of intoxication by the toxic peptide from *M. aeruginosa* NRC-1 have been described previously (7) and resemble those of certain neurotoxins. However, it should be pointed out that mice injected with a lethal dose of the pure toxin invariably died within 1½ hours and most of them within 50 minutes. This provides additional proof (7) that the FDF and SDF produced by this strain of *Microcystis* are different toxins. No evidence of SFD was found in the present work even though the algae were contaminated with bacteria suspected of being the source of this factor (7, 20).

Among naturally occurring peptides there are a number of toxic substances and some of these which are toxic to animals also display antibiotic activity. On the basis of our present knowledge it is difficult to generalize about relations between structure and biological activity of naturally occurring peptides. However, examination of a review of naturally occurring peptides (21) has revealed that most of those having antibiotic activity are "heteromeric" peptides, i.e. they are made up of amino acids plus one or more other molecules, e.g. actinomycin. In this connection it is interesting that the peptide studied here was "homeomeric", i.e. made up only of amino acids, and although toxic to animals displayed no antibiotic activity.

It has been noted that biologically active peptides of natural occurrence possess structural features that are abnormal to the "ordinary" protein (21, 22). Among these features may be an abnormal optical configuration (D- instead of L-), an amino acid of unusual occurrence, or a cyclic structure.

The toxic peptide from *M. aeruginosa* NRC-1 possesses all of these "unusual" features in having D-serine, L-ornithine, and a cyclic structure. However, it is difficult to attribute the toxicity to any or all of these features with any degree of certainty. A recent article by Woolley and Merrifield (23) has shown that the requirements of occurrence and arrangement of amino acids in biologically active peptides are not nearly as specific as has generally been supposed. Clearly more work is required to relate structure to biological activity in the naturally occurring peptides. The isolation of five closely related peptides, only one of which is toxic, from *M. aeruginosa* NRC-1 appears to offer a unique opportunity for the study of this relationship.

Acknowledgments

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References

1. Fitch, C. P., Bishop, L. M., and Boyd, W. L. Cornell Vet. **24** (1), 30 (1934).
2. Wheeler, R. E., Lackey, J. B., and Schott, S. Public Health Repts. **57** (45), 1695 (1942).
3. Louw, P. G. J. S. African Ind. Chemist, **4**, 62 (1950).
4. Shelubsky, M. Proc. Intern. Assoc. Limnology, **11**, 362 (1951).
5. Stewart, A. G., Barnum, D. A., and Henderson, J. A. Can. J. Comp. Med. Vet. Sci. **14**, 197 (1950).
6. Olson, T. A. Proceedings of inservice training course in water works problems. The University of Michigan, Ann Arbor, Mich., Feb. 15 and 16, 1951 (Mimeographed bulletin).
7. Hughes, E. O., Gorham, P. R., and Zehnder, A. Can. J. Microbiol. **4**, 225 (1958).
8. Reindel, F. and Hoppe, W. Chem. Ber. **87**, 1103 (1954).
9. Foster, A. B. Chem. & Ind. 1050 (1952).
10. Briggs, D. R., Garner, E. F., and Smith, F. Nature, **178**, 155 (1956).
11. Mitchell, H. K. and Herzenberg, L. A. Anal. Chem. **29**, 1229 (1957).
12. Albon, N. and Gross, D. Analyst, **77**, 510 (1952).
13. Blackburn, S. and Lowther, A. G. Biochem. J. **48**, 126 (1951).
14. Levy, A. L. Nature, **174**, 126 (1954).
15. Jones, T. S. G. Biochem. J. **42**, LIX (1948).
16. Sanger, F. Biochem. J. **39**, 507 (1945).
17. Cooper, D. R. Nature, **181**, 713 (1958).
18. Consden, R., Gordon, A. H., Martin, A. J. P., and Synge, R. L. M. Biochem. J. **41**, 596 (1947).
19. Sanger, F. Advances in Protein Chem. **VII**, 21 (1952).
20. Thomson, W. K., Laing, A. C., and Grant, G. A. Rept. No. 51, Defence Research Board, Canada, September, 1957.
21. Bricas, E. and Fromageot, C. Advances in Protein Chem. **VIII**, 1 (1953).
22. Synge, R. L. M. Roy. Inst. Chem. Lectures, Monographs Repts. No. 1 (1952).
23. Woolley, D. W. and Merrifield, R. B. Science, **128**, 238 (1958).

COMPARISON BETWEEN SEASONAL AND THERMAL ACCLIMATION IN WHITE RATS

I. METABOLIC AND INSULATIVE CHANGES[1]

O. HÉROUX, F. DEPOCAS, AND J. S. HART

Abstract

Physiological adjustments to cold temperature have been compared in white rats exposed either to the outdoor fluctuating environmental conditions or to the indoor constant temperature conditions. While the metabolic adjustments such as increased peak metabolism and decreased shivering were similar in outdoor and indoor rats exposed to cold, the adjustments in insulation and thermoneutral metabolic rates were quite different. The pelage insulation increased in the rats kept outside during the winter but remained unchanged in the rats kept in a constant temperature room maintained at 6° C. The resting metabolic rate measured at 30° C increased in the 6° C acclimated rats but not in the winter-exposed animals. Over the temperature range +30° C to −15° C, while the indoor cold-acclimated rats had a higher metabolic rate than their controls acclimated to 30° C, the winter rats had a lower metabolism than their summer controls.

Acclimatization to winter conditions in small mammals has often been found to be associated with an increase in pelage insulation. White-footed mice (1) and rabbits (2) have been shown to have a greater pelt weight, and deer mice (3) have denser fur and their pelage insulation is greater during winter (4) than during summer. Further evidence for such an increase in insulation is the reduced heat production observed during the winter in different species including white rats (5) and mice (4) kept outdoors.

In white rats, acclimated in a constant low-temperature room, no evidence for a greater insulation has ever been found. Actually, cold-acclimated rats have been repeatedly shown to have a greater oxygen consumption than warm-acclimated ones (6), which suggests a reduction in insulation since these animals maintain a normal body temperature.

Apparently, under these fixed temperature conditions, cold acclimation is purely a metabolic phenomenon. Increased cold resistance has been associated with the acquired ability of the cold-acclimated rats to maintain a high rate of heat production for a longer period of time, and with the ability to reach a higher maximal rate of heat production at very low exposure temperatures (7). Moreover, while heat is mostly produced by shivering in non-acclimated rats, it becomes exclusively a non-shivering process after cold acclimation (8).

In an attempt to find whether similar metabolic adjustments would also develop under the fluctuating outdoor environmental conditions, cold resistance, peak metabolism, degree of shivering, and pelage insulation have been measured on white rats kept outdoors in a common cage during summer and winter.

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Experimental Procedures

Animals and Environmental Conditions

Adult male Sprague–Dawley rats were submitted to two different sets of environmental conditions for an average of 82 days: (*a*) in rooms maintained at a constant temperature (30° C and 6° C) and illuminated from 8 a.m. to 8 p.m.; (*b*) outside, exposed to the fluctuating environmental conditions from May 15 to August 13 during the summer and from October 23 to January 23 during the winter.

At 30° C and 6° C, the rats were kept individually in 11 in. × 8 in. × 7 in. wire cages, while outside, 10 rats were kept together on shavings in 36 in. × 18 in. × 15 in. wire cages suspended in a rack. Protection against the sun during the summer and against wind and snow storms during the winter was supplied by a wooden enclosure built around the rack and opened only on one side. The average temperatures, reported by the Dominion Public Weather Office, for the region where the outdoor cages were kept are given in Table I.

TABLE I

Average monthly temperatures during acclimatization as reported by the Dominion Public Weather Office

	May	June	July	Aug.	Oct.	Nov.	Dec.	Jan.
Winter 1955–56					+10° C	+1.5° C	−10° C	−7.7° C
Summer 1956	+10° C	+19° C	+19° C	+19° C				
Winter 1956–57					+9.6° C	+2.0° C	−6° C	−14° C
Summer 1957	+12.8° C	+19.6° C	+20.9° C	+18.8° C				
Winter 1957–58					+ 8.5° C (+10.4° C)	+3.4° C (+6.7° C)	−3.4° C (−3.1° C)	−10.5° C (−9.1° C)

*The temperatures shown in parenthesis have been recorded by a Taylor thermometer which was kept in a cage adjoining the rat cages.

The average weight of the rats at the beginning of the acclimation period was 200 g. At the end of the acclimation period, the average body weights were quite similar in all subgroups. Only the average body weight of the subgroups used for oxygen consumption at +30° C are reported in Table II. Throughout the experiments, they were fed Master Fox Chow and tap water ad libitum. The outdoor winter rats had access to snow all the time and they were supplied with lukewarm water twice a day at 9.00 a.m. and 5.00 p.m.

Methods

Cold resistance was determined by measuring the time to death for each rat kept individually in a cage maintained at −35° C. Food was provided at all times but no water was supplied. While only one 30° C group and one 6° C group were tested, four outdoor groups were tested: summer 1956, summer 1957, winter 1956–57, and winter 1957–58.

The oxygen consumption was measured with an open circuit apparatus over the temperature range +30° C to −6° C (7) and with a low lag, closed circuit apparatus over the −15° C to −45° C range. Each indoor and outdoor group was divided into six or seven subgroups, each of which was used at only one temperature. There were from 6 to 18 rats in each subgroup.

TABLE II

	Indoor rats			Outdoor rats		
	30° C	6° C	Difference	Summer	Winter	Difference
Survival time (log min)	2.09±0.01† (7)	2.76±0.09 (8)	0.67±0.09**	2.25±0.02 (15)	2.64±0.08 (18)	0.39±0.08**
O_2 consumption at 30° C ($ml/hr/bwt^{0.8}$)	22.8±0.6 (10)	26.7±1.1 (10)	3.9±1.2**	22.9±1.3 (8)	21.5±0.6 (12)	1.4±1.5
O_2 consumption at 6° C ($ml/hr/bwt^{0.8}$)	38.2±0.6 (10)	43.4±1.0 (10)	5.1±1.2**	39.5±0.6 (8)	34.5±1.4 (12)	5.0±0.4**
Heart rate at 6° C (beats/min)	426±18.5 (8)	391±7.8 (8)	35±20.1**	444±11 (8)	388±6. (6)	56±14.3**
Increase in E.M.G.+ (microvolts)	+15.6±5.6 (8)*	+1.6±2.4 (8)		+32.4±2.3 (8)**	+6.9±1.7 (6)**	
Pelage insulation (° C/cal/m²/hr)	0.31±0.003 (7)	0.31±0.007 (7)	0	0.27±0.004 (8)	0.36±0.015 (8)	0.09±0.01**
Body weight after acclimation (g)	380 (range 357–414) (10)	304 (range 192–385) (10)		423 (range 359–531) (8)	370 (range 278–429) (12)	

NOTE: + Increase in E.M.G. at 6° C over the level recorded at +30° C. ( ) Number of rats given in parenthesis.
*Statistically significant at the 5% level.
**Statistically significant at the 1% level.
†Standard error.

Methods used for estimating the pelage insulation (9) and for measuring heart rates and electromyograms (E.M.G.) have been previously described (8, 10). Oxygen consumption, E.M.G., and heart rates obtained incidentally to the E.M.G. were measured on the same summer or winter subgroups at each test temperature. For the indoor rats, survival time, pelage insulation, E.M.G., and heart rates were measured on rats acclimated for 82 days while oxygen consumption was recorded on rats acclimated for 28 days only. Preliminary experiments had shown, however, that survival time, E.M.G., heart rate, and oxygen consumption were essentially the same after 28 or 82 days of acclimation.

Results

Survival Time

Since there was no apparent difference in average and in variation of log survival time between the two summer groups nor between the two winter groups, these were pooled into one summer and one winter group for comparisons. Log survival times of winter and 6° C rats were extremely variable as compared with those of summer and 30° C rats (Table II). Winter and 6° C rats were much more resistant to low temperatures than summer and 30° C rats. There was no apparent difference in log survival time between winter and 6° C animals.

Metabolism

As reported in a previous paper (7), at 30° C, heat production of 6° C acclimated rats was higher than in 30° C rats (Table II). No significant difference could be observed, however, between the resting metabolism at 30° C of summer and winter rats. Over the temperature range +30° C to −15° C, while the 6° C rats had a higher oxygen consumption than the 30° C rats, the situation was reversed with the outdoor rats; the winter rats had a lower oxygen consumption than the summer ones (Fig. 1). Both 30° C and summer rats reached a maximum metabolic rate between −10° C and −30° C while 6° C and winter rats could still increase their metabolism on further lowering of environmental temperature.

Heart Rates

Over the temperature range +18° C to −6° C, heart rates tended to be higher in 30° C rats than in 6° C rats but the difference was statistically significant only at +6° C (Table II). In the outside rats, heart rates were significantly higher in the summer rats except when measured at +30° C (Fig. 1).

Electromyograms

Over the −6° C to +30° C temperature range, the electrical activity of muscle (E.M.G.) was always greater in summer and 30° C acclimated rats than in winter and 6° C acclimated ones. In both warm groups, the average

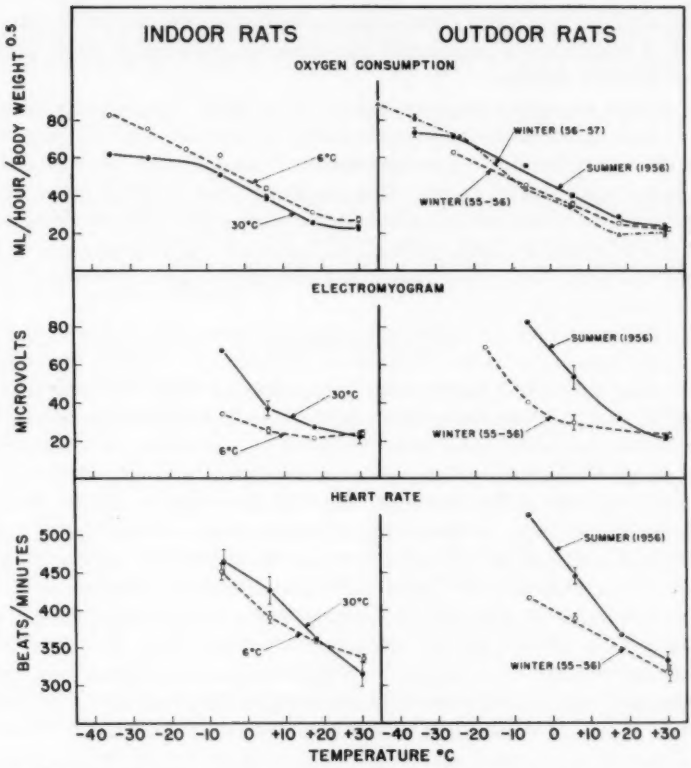

FIG. 1. Oxygen consumption, electromyograms, and heart rates of 30° C and 6° C acclimated rats and summer- and winter-acclimatized rats. Data for oxygen consumption of indoor rats were recalculated from previous results reported by Depocas *et al.* (7).

electrical activity started to increase as soon as the environmental temperature was lowered below +30° C. In the 30° C acclimated rats, the level of E.M.G. obtained at 6° C was 72% greater than the level recorded at 30° C and in the summer rats it was 152% greater. In the 6° C acclimated rats, no significant change was observed at 6° C (Table II) while in the winter rats a 32% increase, which was significant at the 1% level, was obtained.

Pelage Insulation

Pelage insulation was significantly greater in winter than in summer rats (Table II) but no difference was found between the 30° C and the 6° C acclimated rats.

Discussion

In this study, the indoor and outdoor environmental conditions were so different that it is not considered appropriate to make detailed comparisons between the control summer group and the 30° C group or the winter group

and the 6° C group. The comparisons will be limited to the trends in the changes of the different parameters measured on the animals when they were kept outdoors or indoors.

The winter outdoor rats, exposed to an average temperature of around −10° C and occasionally to temperatures as low as −30° C, developed a degree of cold resistance equivalent to that found in rats kept at 6° C. In both indoor and outdoor rats, this similar increased cold resistance was accompanied by enhanced non-shivering thermogenesis and also by enhanced ability (*a*) to maintain heat production, for longer periods of time as shown by longer survival time, (*b*) to reach a higher rate of heat production at very low exposure temperature.

While the metabolic capabilities appeared to be similar in both groups, the metabolic adjustments were different at least in one respect; the cold-acclimated indoor rats had a higher resting metabolism than their warm controls at +30° C, but not the winter rats as compared with the summer animals. These results are in variance with Bargeton's observation of a higher metabolism at 29.5° C (5) in winter rats as compared with the controls. One explanation for this difference might be that our controls to the winter rats were summer rats kept outdoors and exposed to similar fluctuating environmental conditions while Bargeton's controls were rats kept indoors and exposed to a constant 29.5° C with artificial light from 7 hours to 19 hours.

These results show that the laboratory white rats, when exposed to the outdoor winter environmental conditions, behave like the wild animals previously mentioned (1–4) as far as a lower oxygen consumption and a greater insulation are concerned, even though supplied with a constant diet and captive in a cage. While the metabolic adjustments such as increased peak metabolism and decreased shivering are similar in indoor and outdoor rats exposed to cold, the adjustments in insulation and thermoneutral metabolic rate are quite different.

The physiological changes described above indicate that seasonal acclimatization of the white rat differs in certain respects from acclimation to constant thermal environment. Other differences in these two processes will be described in later papers.

References

1. SEALANDER, J. A. Am. Midland Naturalist, **46**, 257 (1951).
2. MAYER, A. and NICHITA, G. Ann. physiol. physicochim. biol. **5**, 621 (1929).
3. HUESTIS, R. R. J. Mammal. **12**, 372 (1931).
4. HART, J. S. and HÉROUX, O. Can. J. Zool. **31**, 528 (1953).
5. BARGETON, D. J. physiol. **46**, 234 (1954).
6. HART, J. S. Rev. can. biol. **16**, 133 (1957).
7. DEPOCAS, F., HART, J. S., and HÉROUX, O. J. Appl. Physiol. **10**, 393 (1957).
8. HART, J. S., HÉROUX, O., and DEPOCAS, F. D. J. Appl. Physiol. **9**, 404 (1956).
9. HART, J. S. Can. J. Zool. **34**, 53 (1956).
10. HÉROUX, O., HART, J. S., and DEPOCAS, F. J. Appl. Physiol. **9**, 399 (1956).

AGGRAVATION OF CHOLESTEROL ATHEROSCLEROSIS IN RABBITS BY FREE UNSATURATED FATTY ACIDS[1]

GEORGE RONA, CLIFFORD I. CHAPPEL, AND ROGER GAUDRY

Abstract

An unsaturated fatty acid preparation containing 90 to 95% linoleic acid was tested on cholesterol-induced hypercholesterolemia and atherosclerosis in the rabbit. The rabbits, treated with cholesterol dissolved in the unsaturated fatty acid, revealed a significantly higher hypercholesterolemia and more marked atherosclerosis than the rabbits that were treated with cholesterol dissolved in the same amount of olive oil.

Introduction

Dietary measures may influence serum cholesterol levels of human subjects and thus there is a hope that by such means the development of atherosclerosis may be influenced. It was shown by Kinsell, Margen, and Michaels (10) that the ingestion of certain vegetable fats under precisely controlled conditions caused a sustained fall in the plasma cholesterol and phospholipid levels. Opinions differ, however, as to which factor in the vegetable fat lowers the blood cholesterol. According to Kinsell and Michaels (11) and Kinsell, Michaels, Friskey, and Splitter (12) the vegetable fats exert their influence by virtue of their unsaturated fatty acid content. Beveridge and his colleagues (2, 3, 4) suggested that the plasma cholesterol depressant action depends upon the sterol content of these oils. Because of the great practical value of these questions (Ahrens *et al.* (1)) it seemed interesting to investigate this problem in animal experiments.

Experimental

An unsaturated fatty acid preparation, referred to as "9-11 Acids"* was tested on experimental atherosclerosis in the rabbit. "9-11 Acids" assays between 90–95% linoleic acid, of which approximately 30% is in the conjugated 9,10-11,12 form, and approximately 70% is in the more common non-conjugated 9,10-12,13 form. The "9-11 Acids" also contained 4 to 5% of oleic acid, 2 to 3% saturated C-18 acids, and traces of ricinoleic acid. The acid value is 197, iodine value 153, and saponification value 198. The compound is insoluble in water and soluble in most organic solvents. It is a clear, light, low-viscosity liquid.

The animals used for this experiment were 40 albino rabbits. The groups were composed of equal numbers of males and females with average starting weights of 2400 and 2200 respectively. The rabbits were fed commercial rabbit food (Purina) supplemented with cabbage. Before the experiment

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Contribution from Research Laboratories, Ayerst, McKenna & Harrison Ltd., Montreal, Que.

*We are indebted to Baker Castor Oil Co., 120 Broadway, New York 5, N.Y., for generous supplies of this material.

was begun, and each week during the study, blood was taken from the ear vein for serum cholesterol and lipid phosphorus determination. All rabbits were bled on the same day, after an overnight fast, and prior to the administration of cholesterol. Pearson, Stern, and McGavack's method (15) was used for total serum cholesterol determination and Youngburg's method (18) was used for phosphorus extractions from the serum. The method of King (9) was used for phosphorus determination. Atherosclerosis was produced by giving cholesterol by stomach tube. The amount of cholesterol introduced was 0.5 g/kg every second day. This amount of cholesterol was dissolved in 5 ml of "9-11 Acids" in the experimental, and 5 ml of olive oil, in the control group. The cholesterol – olive oil mixture was a partial suspension, while the cholesterol in "9-11 Acids" formed a clear solution. The treatment lasted 4 weeks, then the cholesterol dosage was discontinued for 3 weeks in order to study the regression of the hypercholesterolemia.

At the end of the experiment, which lasted 7 weeks, the rabbits were sacrificed, autopsies performed, and the weight of heart, liver, and spleen determined. The aorta was taken out with the heart and after formalin fixation was stained for 12 hours with Sudan IV for macroscopic determination of cholesterol deposition. The lesions of the aorta were examined and graded in the following manner:

Grade 0 : no lesion detectable.

Grade 1 : scattered tiny spots of cholesterol deposition, localized mainly around the origin of the branches and in the *arcus*.

Grade 2 : distributed, flat, usually small spots, which might be confluent.

Grade 3 : large, confluent, elevated patches.

Results

Except during the first week of cholesterol treatment, the animals ate well. The weight gain of the control animals was greater than that of the "9-11 Acids" treated rabbits. During the 6-week experimental period the average weight gain was approximately 400, 800, 700, and 1000 g in the treated male, control male, treated female, and control female groups respectively. A transient diarrhoea was observed in some of the "9-11 Acids" treated animals. One rabbit in the treated female group died due to bronchopneumonia.

Table I summarizes the serum cholesterol levels of the "9-11 Acids" and olive oil treated groups during the experiment. The results demonstrate that both males and females that received the cholesterol dissolved in "9-11 Acids" had a higher serum cholesterol level than the controls. This was significantly higher at the 95% level of confidence after the second week of treatment in the female and after the fourth week in the male group. A significant difference was also observed after the cholesterol treatment was discontinued.

Table II indicates that the cholesterol treatment caused an elevation of the serum lipid phosphorus. This elevation was more marked in the "9-11 Acids" treated group with significance at the 95% level of confidence.

TABLE I

Comparison of serum cholesterol levels in the rabbit when cholesterol was given orally together with olive oil or "9-11 Acids"

Group	Sex	Pretreatment	Treatment, weeks 1	2	3	4	Posttreatment, weeks 5	6	7
Control	Male	130 ±11*	356 ±70	518 ±100	777 ±207	930 ±240	588 ±160	492 ±184	255 ±80
"9-11 Acids"	Male	133 ±16	478 ±20	813 ±135	1143 ±280	1510 ±445	1238 ±310	1176 ±200	752 ±200
Control	Female	168 ±20	417 ±67	564 ±66	843 ±60	776 ±80	859 ±82	465 ±189	295 ±146
"9-11 Acids"	Female	157 ±45	461 ±28	948 ±64	1505 ±60	2071 ±138	1806 ±153	1666 ±110	1190 ±288

*Values are expressed in mg% with the standard error of the mean.

TABLE II

Comparison of serum lipid phosphorus values in the rabbit when cholesterol was given orally together with olive oil or "9-11 Acids"

Group	Sex	Pretreatment	Treatment, weeks 1	2	3	4	Posttreatment, weeks 5	6	7
Control	Male	4.2* ±0.6	7.6 ±1.2	8.8 ±1.7	10.6 ±2.3	12.4 ±2.8	9.9 ±1.8	8.9 ±2.2	6.5 ±1.1
"9-11 Acids"	Male	4.7 ±1.1	9.5 ±0.3	13.4 ±4.2	13.5 ±2.4	19.9 ±4.2	19.3 ±3.0	18.3 ±1.6	12.2 ±2.0
Control	Female	±4.1 ±0.8	11.2 ±3.7	—	14.8 ±2.0	14.3 ±1.7	15.6 ±2.0	10.4 ±2.4	8.2 ±1.9
"9-11 Acids"	Female	4.8 ±0.8	9.9 ±0.9	26.4 ±9.8	17.3 ±0.3	24.7 ±1.3	25.6 ±1.2	24.2 ±1.4	31.7 ±3.0

*Values are expressed in mg% with the standard error of the mean.

TABLE III

Comparison of cholesterol/lipid phosphorus ratios in rabbit when cholesterol was given together with olive oil or "9-11 Acids"

	Pretreatment	Treatment, weeks 1	2	3	4	Posttreatment, weeks 5	6	7
Control*	35.6 ±6.6†	42.0 ±6.5	58.4 ±4.8	73.3 ±9.5	64.5 ±8.1	57.0 ±7.4	49.8 ±7.0	37.5 ±7.8
"9-11 Acids"	30.1 ±7.4	48.3 ±4.2	48.3 ±9.8	85.6 ±5.8	79.7 ±6.6	67.2 ±5.1	66.6 ±6.5	46.8 ±6.4

*In this table values for both males and females are pooled.
†Standard error of the mean.

As Table III shows, the cholesterol/lipid phosphorus ratio increased during the cholesterol treatment. It declined again when cholesterol administration was stopped. The "9-11 Acids" treated group had a higher ratio from the third week of experiment due to the marked elevation of the serum cholesterol levels. This difference was not significant. In the "9-11 Acids" treated group, the cholesterol/lipid phosphorus ratio remained above the initial level until the end of the experiment.

Autopsy revealed coccidiosis of the livers of three females, one of which belonged to the "9-11 Acids" treated and two to the control group. The weights of the spleen, liver, and heart of the animals from the two groups were not significantly different. Examination of the aortas revealed atherosclerosis which was mild or moderate in severity in all groups. Table IV shows the

TABLE IV

Grade of cholesterol deposition in the aorta

Males		Females	
"9-11 Acids"	Control	"9-11 Acids"	Control
1.4 ± 0.2*	0.8 ± 0.2	2.8 ± 0.1	1.2 ± 0.2

*Standard error. Differences between "9-11 Acids" treated and control groups are significant at the 95% level of confidence.

mean grade of cholesterol deposition in the aorta of the different groups. We observed more marked aortic atherosclerosis both in males and females of the "9-11 Acids" treated group. These differences were statistically significant. It was interesting to note that in our experiment the females reacted with a more pronounced atherosclerosis than the males. There was a direct relationship between the serum cholesterol levels and the severity of aortic atherosclerosis.

Discussion

Our experiment showed that a preparation containing free unsaturated fatty acids had no preventive effect on the development of cholesterol-induced hypercholesterolemia and atherosclerosis in the rabbit. The unsaturated fatty acids had, on the contrary, a deleterious action. Rabbits treated with cholesterol dissolved in "9-11 Acids" revealed a significantly higher cholesterolemia and a more marked atherosclerosis than the rabbits which received cholesterol dissolved in a similar amount of olive oil.

There is a limit to the conclusions which may be drawn from these studies in the rabbit. The irritant properties of the "9-11 Acids" which caused mild diarrhoea in some of the animals may have influenced the absorption of cholesterol. However, such a diarrhoea would not be expected to enhance cholesterol absorption. If the diarrhoea were an important factor it would be expected to lower the cholesterol absorption, whereas in Table I it is shown that the serum cholesterol is higher in the "9-11 Acids" group either during the treatment or posttreatment periods. It is recognized that a form of free saturated fatty acids would have been a more appropriate control substance than olive oil to test the role of unsaturation per se. However, in spite of these limitations in making a comparison between the groups, the fact is established that an unsaturated free fatty acid preparation failed to protect rabbits against cholesterol-induced aortic atherosclerosis.

Our experiments, which show a deleterious effect of an unsaturated fatty acid preparation on serum cholesterol and aortic lesions in the cholesterol fed rabbit, are in agreement with the findings of a number of authors. Swell

et al. (17) have found that unsaturated fats in the diet of rats on a cholesterol-free diet elevated the serum cholesterol levels. This effect was more pronounced if the unsaturated fats were in the form of free fatty acids. Thus it is clear that unsaturated fatty acids would enhance the levels of serum cholesterol derived from endogenous sources. Other studies have shown that the absorption of cholesterol was also improved when unsaturated fats were present in the diet, whereas the absorption of cholesterol became incomplete when a high percentage of saturated fatty acids were fed (16).

Kritchevsky *et al.* (13) have found in rabbit experiments similar to ours that cholesterol deposition in the aorta was more marked in animals receiving cholesterol in partially hydrogenated vegetable fat than in those which received cholesterol administered in corn oil. The same group (14) in a more recent paper reported that when methyl esters of stearic, oleic, and octa-9-11 dienoic acids were used as vehicles for cholesterol, the rabbits receiving the most unsaturated ester, that of octa-9-11 dienoic acid, had the highest serum cholesterol levels. Keys *et al.* (7, 8) and Hegsted *et al.* (5) have shown that a correlation cannot be drawn between the effect of certain fatty acids on serum cholesterol in man and their degree of unsaturation. These authors believe that a complementary effect is obtained by combining saturated and unsaturated fat in the diet.

It is clear that the role of unsaturation in fats or fatty acids on the development of experimental atherosclerosis has yet to be definitively outlined. Meanwhile it would appear that the statement "When vegetable fat is fed (because it is unsaturated) there is a fall in cholesterol and phospholipid values, and when animal fat is fed (because it is saturated) we find a rise in these substances" (6)—may perhaps be an oversimplification.

References

1. Ahrens, E. H., Jr., Hirsh, J., Insull, W., Jr., Tsaltas, T. T., Blomstrand, R., and Peterson, M. L. Lancet, **II**, 943 (1957).
2. Beveridge, J. M. R., Connell, W. F., and Mayer, G. A. Circulation, **10**, 593 (1954).
3. Beveridge, J. M. R., Connell, W. F., and Mayer, G. A. Can. J. Biochem. and Physiol. **35**, 257 (1957).
4. Beveridge, J. M. R., Connell, W. F., Mayer, G. A., and Haust, H. L. Can. J. Biochem. and Physiol. **36**, 895 (1958).
5. Hegsted, D. M., Gotsis, A., and Stare, F. J. Circulation, **16**, 479 (1957).
6. Heller, E. M. Can. Med. Assoc. J. **77**, 895 (1957).
7. Keys, A., Anderson, J. T., and Grande, F. Lancet, **II**, 959 (1957).
8. Keys, A., Anderson, J. T., and Grande, F. Lancet, **I**, 742 (1958).
9. King, E. J. Microanalysis in medical biochemistry. J. & A. Churchill, Ltd., London. 1951. p. 65.
10. Kinsell, L. W., Margen, S., and Michaels, G. D. J. Clin. Endocrinol. **12**, 909 (1952).
11. Kinsell, L. W. and Michaels, G. D. Am. J. Clin. Nutrition, **3**, 247 (1955).
12. Kinsell, L. W., Michaels, G. D., Friskey, R. W., and Splitter, St. Lancet, **I**, 334 (1958).
13. Kritchevsky, D., Moyer, A. W., Tesar, W. C., Logan, J. B., Brown, R. A., Davies, M.C., and Cox, H. R. Am. J. Physiol. **178**, 30 (1954).
14. Kritchevsky, D., Moyer, A. W., Tesar, W. C., McCandless, R. F., Logan, J. B., Brown, R. A., and Englert, M. E. Am. J. Physiol. **185**, 279 (1956).
15. Pearson, S., Stern, S., and McGavack, T. H. Anal. Chem. **25**, 813 (1953).
16. Swell, L. and Flick, D. F. Am. J. Physiol. **174**, 51 (1953).
17. Swell, L., Flick, D. F., Field, H., Jr., and Treadwell, C. R. Am. J. Physiol. **180**, 124 (1955).
18. Youngburg, G. E. and Youngburg, M. V. J. Lab. Clin. Med. **16**, 158 (1930).

VITAMIN B_6 AND MENTAL DEFICIENCY

THE EFFECTS OF LARGE DOSES OF B_6 (PYRIDOXINE) IN PHENYLKETONURIA[1]

EDITH G. MCGEER AND BLUMA TISCHLER

Abstract

Vitamin B_6 was given to 10 phenylketonuric patients in doses up to 150 mg/day for a period of 9 weeks. Their clinical signs, behavior, intelligence, E.E.G.('s), serum phenylalanine levels, urinary phenylpyruvic acid and phenol levels, and excretions of ethyl-acetate-soluble aromatic derivatives were followed serially. No changes of significance to phenylketonuria were found, although chromatographic evidence indicated some changes in excretion of a few aromatic derivatives, notably N^{α}-acetyltryptophan.

Introduction

It is well known that in phenylketonuria there is a blockage of the normal conversion of phenylalanine to tyrosine and a consequent accumulation of phenylalanine in the blood. Abnormal products such as phenylpyruvic acid, *o*- and *m*-tyrosine, and other phenols appear in the urine (Fig. 1). This metabolic error seems to be causally related to the mental deficiency although the exact pathogenesis is not known. Since vitamin B_6 is generally concerned in transaminations, the constant demand for this vitamin in the conversion of phenylalanine to phenylpyruvic acid might result in a relative B_6-deficiency in phenylketonuric patients. Some of the symptoms of pyridoxine deficiency, such as convulsions and dermatitis, resemble those seen in many phenylketonurics in the younger age group; the low levels of 5-hydroxytryptamine reported for phenylketonurics (1) are also consistent with a B_6-deficiency since B_6 is also a coenzyme in the synthesis of 5-hydroxytryptamine from tryptophan. However, no evidence was found of excessive excretion of xanthurenic acid by seven phenylketonurics as compared with six other mentally deficient children, either before or after a tryptophan load (2); such excretion is generally regarded as a very sensitive test of B_6 deficiency.

Despite these negative results, we decided to test the chemical and clinical effects of supplementation with large doses of B_6 in phenylketonuria. Such supplements might conceivably stimulate the conversion of phenylalanine to phenylpyruvic acid, and thereby decrease not only the phenylalanine levels in the blood but also the amount transformed into abnormal phenolic derivatives. If the formation of phenylpyruvic acid is a detoxication reaction, as seems possible from its occurrence in the kidneys, such stimulation might be

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Contribution from the Woodlands School, New Westminster, B.C. (Residential School for Retarded Children), and the Department of Neurological Research, University of British Columbia, Vancouver, B.C. The work of one of us (E.G.McG.) was supported by a Federal Mental Health Grant.

of clinical benefit. We were also encouraged to try the effect of B_6 loads by Bessey's (3) report of the occurrence of B_6-dependent convulsions in some infants who do not excrete excessive xanthurenic acid.

Materials and Methods

(a) Clinical

The 10 phenylketonurics studied ranged in age from 8 to 41 years and in intelligence from idiot to moron level. All were on a normal diet since phenylketonurics of this age group do not appear to be helped by a phenylalanine-restricted diet. Seven of the 10 had been tested for B_6-deficiency with negative results (2). Each received supplemental B_6 for a period of 9 weeks; the dose was increased gradually over the first 5 weeks in order to guard against possible toxic side effects. Children under 15 years of age were started on 50 mg of pyridoxine every other day; this was increased to 50 mg/day on the 23rd day and to 150 mg/day after 5 weeks. Adults were started on 50 mg/day, changed to 100 mg/day on the 23rd day, and to 150 mg/day after 5 weeks. These doses seemed more than adequate in view of reports in the literature that convulsions and excessive xanthurenic acid excretion in B_6-deficient infants can be controlled with only 1.0 to 1.4 mg/day (3), that xanthurenic acid excretion in pregnant women can be reduced to normal by 5 mg of B_6 (4, 5), and that 5 to 200 mg of B_6 are sufficient to control the severe deficiency signs brought about by administration of desoxypyridoxine, a vitamin B_6 antimetabolite (6).

A complete physical and neurological examination was done each week. E.E.G. recordings were made before and after 8 weeks of supplementation. Changes in the neurological manifestations, skin disorders, and convulsions shown by these patients were looked for particularly, since polyneuritis, dermatitis, and convulsions have all been reported as signs of B_6 deficiency.

(b) Chemical

Serum phenylalanine levels were determined by the method of Kappeller-Adler (7) at the start of the experiment and after 1, 2, 3, 4, 6, 8, and 9 weeks of B_6 supplementation. Urinary levels of phenylpyruvic acid and of free and total "phenols" were determined at the same times; the former by the method of Penrose and Quastel (8) and the latter by the Folin–Denis procedure (9). The estimations were done on single morning specimens as 24-hour collections are rather difficult with this type of patient. Since creatinine measurements on phenylketonuric urine by Jaffe's method have not been satisfactory in our hands (cf. 10), specific gravity was used as a rough measure of urine concentration.

The excretion of ethyl-acetate-soluble aromatic derivatives was also studied chromatographically. Portions of the urines of each person collected just before B_6-supplementation began, after 4 weeks, after 7 weeks, and at the close of the experiment were acidified to pH 2 with hydrochloric acid, salt-saturated, and extracted repeatedly with ethyl acetate. Aliquots of the

concentrated extracts corresponding to 0.75 and 1.50 cc of urine of specific gravity 1.014 were chromatographed in Armstrong's solvent systems (11), in descending isopropanol/ammonia/water (8:1:1) and *n*-butanol/acetic acid/water (4:1:1) (12), and according to Jepson's technique (13). All chromatograms were examined under ultraviolet light. Armstrong-type chromatograms were then developed with diazotized sulphanilic acid, Jepson-type chromatograms were sprayed with Ehrlich reagent, and both developing agents were used with chromatograms prepared in the descending solvent systems. Compounds revealed on the chromatograms were identified by comparison with the data of Armstrong *et al.* (11), Jepson (13), and Acheson *et al.* (14) and, in doubtful cases, with standards.

Experimental Results

(a) Clinical

No behavioral or intellectual changes were noted during B_6-supplementation.

Nine of the patients had hyperreflexia; four had so-called Parkinsonian features (short-stepped or propulsive gait with decrease of associated movement of the hands and mask-like face); and one patient had digital mannerisms. All of these signs are said to occur frequently in phenylketonurics (15, 16) and none showed any change during the experiment.

Two of our patients had follicular keratosis of the skin and three had dry skin. No changes in these conditions were noted.

As expected for phenylketonurics (3, 17), the majority of our patients showed abnormal E.E.G. patterns. Seven showed paroxysmal cerebral dysrhythmia which was accompanied in two cases by focal accentuation. The other three patients had borderline normal E.E.G.'s although photic

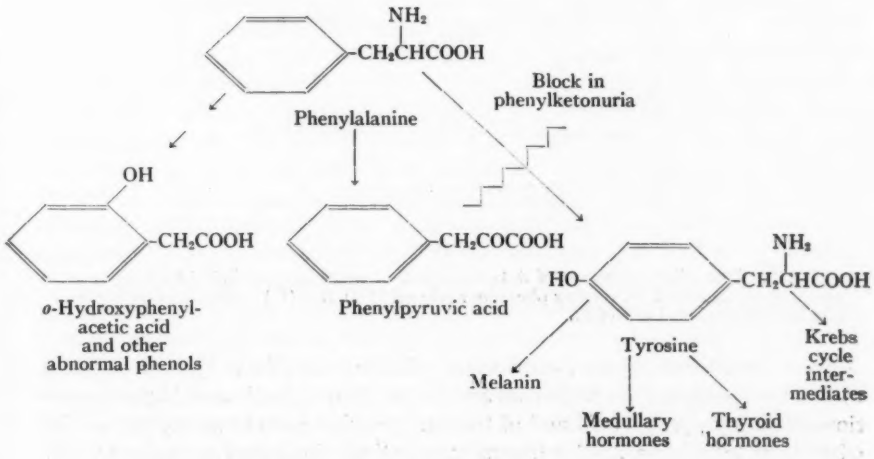

FIG. 1. Primary metabolic block in phenylketonuria.

stimulation and hyperventilation provided an indication of relative instability. The E.E.G.'s taken after 8 weeks of B_6-supplementation were reported as unchanged in seven, and in three they were described as presenting a more active picture. These results are regarded as insignificant since serial E.E.G.'s on our group of phenylketonuric dietary controls showed spontaneous variations (18).

Although four of our patients had a history of seizures and two still occasionally show them, the infrequency of these attacks makes it difficult to decide whether B_6 played any part in their control.

(b) Chemical

The quantitative data for serum phenylalanine and urinary phenylpyruvic acid and phenols indicate a large variation from time to time in a single individual and even greater differences between various individuals. There appeared to be no significant, consistent change in any of these quantities during the experimental period. To illustrate this, the individual averages were calculated and the data expressed as percentages of these averages. Percentage figures for the group were averaged for each time of measurement and the mean figures plotted as a moving average against time (Fig. 2). It is evident from graphs C, D, and E that the urinary phenols show no great over-all change. Serum phenylalanine levels tended to increase slightly and urinary phenylpyruvic acid levels tended to decrease slightly; these changes are not statistically significant.

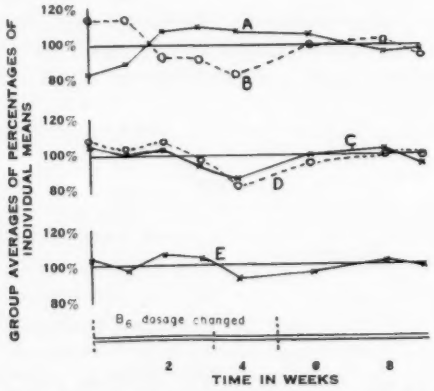

FIG. 2. Plot of group means of data expressed as percentage of individual averages: serum phenylalanine (A), urinary phenylpyruvic acid (B), free (C) and total (D) phenols, and total:free phenol ratio (E).

From the average figures for the various individuals (Table I), there appears to be some tendency for higher serum phenylalanine levels and higher excretions of phenylpyruvic acid and of free and total phenols in phenylketonurics with lower intelligence. The inverse correlations, indicated by these trends, are not statistically significant in this small group.

TABLE I

Sex, physical and mental age, and averaged chemical data for the phenylketonuric subjects studied

Sex	Age, years	I.Q. or Mental age	Serum phenylalanine, mg%	Average values (in mg/cc of urine of sp. gravity 1.010) for urinary: Phenylpyruvic acid	Free phenols	Total phenols
F	13	— 5 months	24	100	0.27	0.38
M	10	— 5 months	24	129	0.19	0.31
F	15	— 2 years	21	92	0.29	0.43
M	9	— 17 months	27	104	0.21	0.27
M	8	28 —	21	76	0.23	0.32
M	11½	30 —	24	129	0.19	0.31
M	17	31 —	23	61	0.24	0.33
M	17	40 —	25	78	0.19	0.28
M	41	63 —	20	70	0.17	0.26
F	35	66 —	16	25	0.12	0.18

There is no significant correlation ($r<0.02$) for the 80 pairs of phenylalanine and phenylpyruvic acid data taken as a whole; for each individual the phenylalanine level shows some tendency to rise as the phenylpyruvic acid excretion falls (cf. curves A and B) but this inverse correlation is also not statistically significant.

The chromatographic pictures obtained resembled those reported for phenylketonurics by Armstrong and others. When the initial (control) chromatograms for each individual are compared with those made from urines collected after B_6-supplementation, no particular change in size or intensity is apparent in the spots given by *o*-hydroxyphenylacetic acid, the most prominent aromatic metabolite in phenylketonuria. There are also no consistent changes evident in the excretion of the majority of other metabolites found commonly in these chromatograms (Table II). Detailed analysis, however, revealed a few changes which seemed to show a consistent trend over the experimental period. The most dramatic was in a compound which was chromatographically similar to N^{α}-acetyltryptophan. This compound has been reported as a human excretory product (14, 19, 20) but normally appears in only very small amounts. Chromatograms on 9 out of 10 urines collected before B_6-supplementation showed a large area corresponding in position and color reactions to N^{α}-acetyltryptophan used as a standard; chromatograms on only 1 of the 10 urines collected after 9 weeks of supplementation showed such a spot. On acid hydrolysis of the extract a new compound appeared, chromatographically similar to both tryptophan and the material formed by acid hydrolysis of synthetic N^{α}-acetyltryptophan. Consistent, but somewhat lesser, increases or decreases were shown by the chromatographic areas given by six (possibly eight) other compounds (Table II). Whether or not these changes are really dependent upon the B_6-supplementation remains unknown. It is tempting to speculate that the drop in excretion of N^{α}-acetyltryptophan may correlate with the slight increase in

TABLE II

Frequency of appearance of compounds in chromatograms of urine before, during, and after B_6-supplementation

No change in frequency	*p*-Hydroxyphenylacetic acid	3-Hydroxyphenylhydracrylic acid
	m-Hydroxyhippuric acid	Salicyluric acid
	m-Hydroxyphenylacetic acid	Homovanillic acid
	p-Hydroxyphenyllactic acid	alpha-Resorcylic acid
	p-Hydroxybenzoic acid	Armstrong No. 24 (11)
	o-Hydroxyphenylacetic acid	Indoxyl sulphate
	Vanillic acid	Indoleacetic acid
	Armstrong No. 18 (11)	Indolelactic acid

	Per cent of chromatograms showing compound for urines collected			
	Before B_6	After 4 wk	After 7 wk	After 9 wk
Increased:				
o-Aminohippuric acid	35%	75%	80%	90%
p-Hydroxyhippuric acid	50	60	65	90
Hippuric acid	60	90	100	100
p-Hydroxymandelic acid	0	30	35	90
p-Hydroxycinnamic acid	20	10	35	70
5-Hydroxyindoleacetic acid	65	70	80	100
Decreased:				
N^{α}-Acetyltryptophan	90	60	20	10
Possibly decreased:				
m-Hydroxyphenylpropionic acid	40	25	20	20
3-Methoxy-4-hydroxymandelic acid	90	25	45	20

excretion of 5-hydroxyindoleacetic acid (the metabolite of 5-hydroxytryptamine) and that both may indicate some degree of "normalization" of tryptophan metabolism. However, the excessive excretion of indoleacetic and indolelactic acids reported for untreated phenylketonurics was found both before and after B_6-supplementation. Further experiments with more rigorously quantitative measures of the excretion of tryptophan metabolites would be necessary before any conclusions could be drawn as to the effect of B_6 loads on tryptophan metabolism in phenylketonurics.

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References

1. PARE, C. M. *et al.* Lancet, **272**, 551 (1957).
2. TISCHLER, B. and McGEER, E. G. Can. Med Assoc. J. **78**, 954 (1958).
3. BESSEY, O. A. *et al.* Pediatrics, **20**, 33 (1957).
4. CHIANCONE, F. M. *et al.* Arch. Biochem. Biophys. **54**, 15 (1955).
5. WACHSTEIN, W. and LOBEL, S. Proc. Soc. Exptl. Biol. Med. **86**, 624 (1954).
6. VITLER, R. W. *et al.* J. Lab. Clin. Med. **42**, 335 (1953).
7. KAPPELLER-ADLER, R. Biochem. Z. **252**, 185 (1932).
8. PENROSE, L. S. and QUASTEL, J. H. Biochem. J. **31**, 266 (1937).
9. FOLIN, O. and DENIS, W. J. Biol. Chem. **22**, 305 (1915).
10. UMBREIT, W. W. and TONHAZY, N. E. Arch. Biochem. Biophys. **32**, 96 (1951).
11. ARMSTRONG, M. D. *et al.* J. Biol. Chem. **218**, 293 (1956).
12. McGEER, E. G. *et al.* J. Nervous Mental Disease, **125**, 176 (1957).
13. JEPSON, J. B. Lancet, **271**, 421 (1956).
14. ACHESON, R. M. *et al.* Can. J. Biochem. and Physiol. **36**, 295 (1958).
15. COWIE, V. J. Mental Sci. **97**, 505 (1951).
16. WRIGHT, S. and TARJAN, G. A.M.A. J. Diseases Children, **93**, 405 (1957).
17. FOIS, A. *et al.* Electroencephalog. and Clin. Neurophysiol. **7**, 569 (1955).
18. TISCHLER, B., DUNN, H. G., *et al.* To be published.
19. LANGNER, R. R. and VOLKMANN, C. M. J. Biol. Chem. **213**, 433 (1955).
20. ARMSTRONG, M. D. *et al.* J. Biol. Chem. **232**, 17 (1958).

THE EFFECT OF UNSATURATION OF DIETARY FAT AND OF ANTIOXIDANTS ON THE DEVELOPMENT OF LIVER DAMAGE[1]

L. S. VALBERG, ROSE A. YOUNG, AND J. M. R. BEVERIDGE

Abstract

The addition of unsaturated fatty acids, even in small amounts, to diets low in vitamin E, selenium, and the sulphur-containing amino acids greatly accelerated the development of acute liver necrosis in rats. The production of this lesion was shown also to be affected in a similar manner by the nature of the dietary fat in the ration consumed immediately prior to the removal of protective substances such as the sulphur-containing amino acids; unsaturated fatty acids or lipid again acted as predisposing factors.

Gamma-tocopherol incorporated in the diet at a level of 0.02% and injected at a level of 1.5 mg/rat/day was equally as effective as alpha-tocopherol in preventing liver necrosis. The protective effect of gamma-tocopherol against the development of liver necrosis lends support to the thesis that alpha-tocopherol protects against liver damage by virtue of its antioxidant activity.

Some years ago reports by Dam *et al.* (1) and by ourselves (2) indicated that dietary fat in the form of lard greatly hastened the development of acute liver damage in rats fed diets low in alpha-tocopherol and in the sulphur-containing amino acids. As Schwarz *et al.* (3) have shown recently, these rations also must have been low in selenium, a newly discovered prophylactic agent closely related to Schwarz's factor 3. Since the inclusion of lard in the diet potentiated the development of hepatic damage, attempts were made in this laboratory to discover the nature of the active material in this fat. Lard was fractionated into unsaponifiable matter, relatively saturated fatty acids, and relatively unsaturated fatty acids, and most of the activity appeared to be in the latter preparation (4). Although unsaturation of fat has been shown to aggravate certain of the manifestations of vitamin E deficiency (1, 5, 6, 7), it seems that no investigation has been performed on this point in connection with the development of acute massive liver necrosis. For this reason the following experiments were carried out.

Experiment 1

The Effect of Type of Fatty Acid on the Development of Liver Necrosis

Eleven groups, each comprised of 10 weanling rats, were given the following fat-free basal diet: Fleischman's active baker's yeast (ground and rendered free from fat by repeated extraction with hot alcohol), 18%; salt mixture, 3%; celluflour, 2%; cod liver oil concentrate, 0.02% (Ayerst, McKenna and Harrison, 50,000 I.U. vitamin D and 200,000 I.U. vitamin A per g); vitamin–sugar mixture, 0.7% (2); choline chloride, 0.3%; sucrose, 75.98%. The fat component of the diet was added at the 10% level in place of an equal

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Contribution from the Department of Biochemistry, Queen's University, Kingston, Ontario.

amount of sucrose and was varied as follows: butyric, caproic, caprylic, capric, lauric, myristic, palmitic, stearic, oleic, linoleic.

The experiment was terminated after 107 days and the surviving animals were killed and autopsied. Diagnosis of liver necrosis was based on both macroscopic and microscopic inspection of the liver.

Results and Discussion

The results of the experiment are shown in Table I and demonstrate clearly that the necrogenic action of linoleic acid is much greater than that of any of the other fatty acids tested.

TABLE I

The effect of fatty acids on the incidence of dietary liver necrosis

Group	Supplement	Incidence of necrosis
I	None	0
II	Butyric acid	0
III	Caproic acid	1/10
IV	Caprylic acid	0
V	Capric acid	0
VI	Lauric acid	0
VII	Myristic acid	0
VIII	Palmitic acid	1/10
IX	Stearic acid	1/10
X	Oleic acid	5/10
XI	Linoleic acid	10/10
XII	Lard	8/10

The group given linoleic acid showed 100% incidence of liver necrosis whereas the others given the saturated fatty acids as the fat component showed a zero incidence or at most 10%. Eighty per cent of the group given the ration containing lard developed acute liver damage. It is reasonable to suppose that the unsaturated fatty acid residues are responsible for the necrogenic effect of lard and, indeed, of fats and oils in general. Furthermore, this effect might be due to the action of peroxides or their breakdown products produced in vitro after the diet was mixed or in vivo after the ration was ingested. In experiment 2 described below further information was obtained on the effect of linoleic acid.

Experiment 2

The Effect of Small Amounts of Linoleic Acid on the Development of Liver Necrosis

Twenty-one weanling rats were fed the fat-free basal diet described in experiment 1 for 109 days and then were divided into three equal groups A, B, and C. Group A was continued on the basal diet and groups B and C were given supplements of 0.25% and 1% peroxide-free linoleic acid. The latter was added to the ration daily.

Results and Discussion

The results of the experiment shown in Table II show that the incidence and average times at which death due to liver necrosis occurred were 7/7 at 10 days and 7/7 at 7.6 days for the groups receiving the 0.25% and 1% linoleic acid diets respectively. Only one out of seven animals on the fat-free regimen developed liver damage. Thus levels of linoleic acid definitely less than that required to supply the estimated daily requirement for essential fatty acids potentiate the development of acute hepatic necrosis.

TABLE II

The effect on the development of liver necrosis of the addition of small amounts of linoleic acid to a fat-free diet

Group	Supplement	No. of animals	No. dying of liver necrosis	Average survival time, days
A	——	7	1	
B	0.25% linoleic acid	7	7	10
C	1.0% linoleic acid	7	7	7.6

Some years ago McLean and Beveridge (2) found that when baker's yeast was extracted with hot alcohol it became much less necrogenic when incorporated into a fat-free diet. Since there was no difference in the effect of the extracted and non-extracted yeast when incorporated into a diet containing lard it was assumed by the authors that the extraction with hot alcohol had removed a necrogenic factor associated with the lipid fraction of the yeast. The lipid comprised about 1.8% of the yeast. Since the experiment reported here has shown that when linoleic acid is added to a fat-free diet in amounts corresponding approximately to the amount of lipid in yeast the development of liver necrosis is greatly accelerated, there would appear to be little doubt that the toxic substance eliminated by the alcoholic extraction of the yeast is unsaturated lipid.

Experiment 3

The Effect of the Antecedent Dietary Fat on the Development of Liver Necrosis

In experiment 1 of this paper it was pointed out that the toxic effect of the unsaturated fatty acids could be due to the production of peroxides and/or their breakdown products in vitro, or in vivo after ingestion of the diet. In the light of the results obtained in the preceding experiments it seemed reasonable to expect that the acute liver damage would develop more readily when the tissues contained a rich supply of highly unsaturated fat. That this type of change in tissue lipid composition can be readily effected in the rat has been demonstrated by Sinclair, who showed that there was a rough parallel between degree of unsaturation of the food and tissue fat (8).

Seventeen weanling rats were fed the fat-free basal diet described above for a period of 109 days in order to deplete the rats of vitamin E and of selenium. They were then transferred to a modified ration high in unsaturated lipid, a

feature designed to ensure depletion of vitamin E reserves. The diet contained 8% linoleic acid, 7% cod liver oil, 40% dried baker's yeast, 0.5% methionine, and 0.25% cystine. The remainder of the diet, except for the carbohydrate moiety, which was appropriately decreased, was the same as for the basal ration. The high level of yeast plus the supplements of methionine and cystine protected the animals even though unsaturated fat in the form of linoleic acid and cod liver oil was in the ration. The animals, indeed, appeared to thrive. After a period of 30 days, two animals were returned to the initial fat-free regimen devoid of supplementary methionine and cystine and containing 18% yeast. The remaining rats were divided into four groups of five each and continued on diets in which various lipids at a level of 5% were substituted for the unsaturated fat in the diet as follows: group A, myristic acid; group B, oleic acid; group C, linoleic acid. After 20 days on these rations the animals were returned to the initial diet, which was free from fat and contained no protective supplements of methionine and cystine.

Results and Discussion

The two animals transferred from the diet high in unsaturated lipid, but high in yeast and supplemented with methionine and cystine to the initial fat-free diet, died of acute liver damage within 36 hours. Obviously the high level of yeast and sulphur-containing amino acids had been affording protection to the animals and the transfer to a diet devoid of supplementary methionine and cystine and containing a lower level of yeast, despite the simultaneous removal of the highly unsaturated lipid from the ration, permitted the acute liver damage to occur. Presumably the lesion developed because of some in vivo change occurring in the unsaturated tissue lipid.

In the case of the animals that had been maintained for a period of 20 days on diets in which the fat was provided in the form of myristic, oleic, or linoleic acids prior to transfer to the initial fat-free diet, although the incidence of death due to massive hepatic necrosis was 100% in all groups, the average survival times varied (cf. Table III). These were for linoleic, oleic, and myristic fed rats respectively, 53, 80, and 133 hours.

TABLE III

The effect of the nature of dietary lipid in antecedent diets on the development of liver necrosis in animals on a fat-free ration

Group	Fat supplement before transfer to a fat-free basal diet	Iodine number of fat	Mortality due to liver necrosis	Average survival time, hours ± S. D.
A	Myristic acid 5%	0	5/5	133±71
B	Oleic acid 5%	59	5/5	80±53
C	Linoleic acid 5%	133	5/5	53±17

These results have been interpreted as being consistent with the theory that the toxic action of unsaturated lipid could be due to some change in this material in vivo, possibly peroxidation.

Experiment 4

The Prophylactic Effect of Gamma-Tocopherol on the Development of Liver Necrosis

The suggestion made by Dam and Granados (1) that alpha-tocopherol protects against the development of liver necrosis by virtue of its antioxidant activity has been refuted by the fact that gamma-tocopherol, a potent antioxidant, has been reported to be ineffective in the prevention of liver damage. Selzer *et al.* (9) reported that the administration of 0.8 mg of gamma-tocopherol to rats by mouth each week afforded no protection against the development of liver necrosis. Similarly Emerson and Dooley (10) found that gamma-tocopherol incorporated into a torula yeast diet had only 1/10th the protective effect of the alpha form.

In view of the findings of Lundberg and co-workers (11), who demonstrated that gamma-tocopherol when administered by mouth was deposited in the tissues in smaller amounts than the alpha form, it appeared to be desirable to reassess the effect of gamma-tocopherol in the development of liver necrosis. In the present study gamma-tocopherol was given both by mouth and by injection to ensure adequate levels in the tissues.

An additional circumstance complicating the interpretation of some of the previously reported studies on the effect of gamma-tocopherol and one which emphasized the need for reassessment of its nutritional role is the fact that some samples, probably most of them, almost undoubtedly contained a significant amount of alpha-tocopherol (12).

Thirty-four weanling rats of the Sprague–Dawley strain were given the basal fat-free diet described above. After 109 days on this regimen the animals were divided into four groups: group A was maintained on the basal diet while the other three groups (B, C, and D) were fed the basal diet supplemented with 10% ethyl esters of safflower oil which had been oxidized at 25° C to a peroxide number of 176 meq/kg; group B received no additional supplement; groups C and D were given respectively alpha- and gamma-tocopherol* at a level of 0.02% in the diet and 1.5 mg by subcutaneous injection each day.

Results and Discussion

The results are shown in Table IV. The animals that received the ethyl esters of safflower oil died of hemorrhagic necrosis of the liver at an average time of 140 hours, whereas only one animal out of seven on the fat-free regimen developed the lesion. Both alpha- and gamma-tocopherol provided complete protection against the development of liver necrosis.

The work of Lundberg and co-workers (11, 13, 14) has demonstrated that there is a selective deposition of the tocopherols in the tissues. The quantities of alpha-, beta-, and gamma-tocopherol in the abdominal fat of vitamin

*The sample of gamma-tocopherol, free from alpha-tocopherol as determined by chromatographic analysis, was provided through the courtesy of Dr. David Herting of the Distillation Products Industries Ltd., Rochester, New York.

TABLE IV

The effect of alpha- and gamma-tocopherol on the development of dietary liver necrosis

Group	Supplement	No. of animals	No. dying of liver necrosis	Average survival time, hours
A	None	7	1	——
B	10% ethyl esters of safflower oil*	9	9	140
C	10% ethyl esters of safflower oil* plus alpha-tocopherol	9	0†	——
D	10% ethyl esters of safflower oil* plus gamma-tocopherol	9	0†	——

*A preparation of ethyl esters of the fatty acids of safflower oil oxidized to a peroxide content of 176 meq/kg. The iodine number was 82.
†One animal died suddenly of unknown cause and without obvious anatomical lesions.

E-deficient rats after a single oral dose of tocopherol was greatest for the alpha form. These results have been confirmed in certain other species, e.g. the cow and the hen (15, 16). This selective deposition of alpha-tocopherol in animal tissues might explain the failure of Selzer *et al.* (9) and Emerson and Dooley (10) to obtain an appreciable protective effect when gamma-tocopherol was included in the diet.

Barnes (17) and Dam *et al.* (18) have demonstrated clearly that the tissue lipids of vitamin E depleted rats are extremely sensitive to oxidation. It is suggested that in the development of acute liver necrosis, a lack of vitamin E permits an auto-oxidation of unsaturated lipid in the tissues. Presumably the breakdown of the unsaturated lipid or the oxidation products produced are responsible for the cellular damage. The nature of the role of selenium and of the sulphur-containing amino acids is not known at present but may be concerned with the preservation of unsaturated lipid or the prevention of the toxic action of the breakdown products.

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References

1. DAM, H. and GRANADOS, H. Acta Pharmacol. Toxicol. **7**, 181 (1951).
2. MCLEAN, J. R. and BEVERIDGE, J. M. R. J. Nutrition, **47**, 41 (1952).
3. SCHWARZ, K., BURI, J. I., BUGGS, C. M., and SCOTT, M. L. Proc. Soc. Exptl. Biol. Med. **95**, 621 (1957).
4. MCLEAN, J. R., BRONSTEIN, P., and BEVERIDGE, J. M. R. Unpublished data.
5. SCHWARZ, K. Ann. N. Y. Acad. Sci. **52**, 225 (1949).
6. DAM, H. J. Nutrition, **27**, 193 (1944).
7. DAM, H. Proc. Soc. Exptl. Biol. Med. **52**, 285 (1943).
8. SINCLAIR, R. G. J. Biol. Chem. **92**, 245 (1931).

9. SELZER, G., PARKER, R. G. F., MCKENZIE, D., and LINDAN, G. C. Brit. J. Exptl. Pathol. **32**, 493 (1951).
10. EMERSON, G. A. and DOOLEY, C. L. Federation Proc. **14**, 431 (1955).
11. LUNDBERG, W. O., BARNES, R. H., CLAUSEN, M., and BURR, G. O. J. Biol. Chem. **153**, 265 (1944).
12. HERTING, D. Personal communication. Distillation Products Industries Limited, Rochester, New York.
13. LUNDBERG, W. O., BARNES, R. H., CLAUSEN, M., LARSON, N., and BURR, G. O. J. Biol. Chem. **168**, 379 (1947).
14. BURR, G. O., LUNDBERG, W. O., and CHIPAULT, J. R. Oil and Soap, **23**, 382 (1946).
15. DJU, M. Y., QUAIFE, M. L., and HARRIS, P. L. Am. J. Physiol. **160**, 259 (1950).
16. MATTILL, H. A. The vitamins. Vol. III. Academic Press, Inc., New York. 1954. p. 501.
17. BARNES, R. H. Biological antioxidants. Trans. of the First Conference. Josiah Macy, Jr. Foundation, New York. 1946. p. 49.
18. DAM, H., KRUSE, I., PRANGE, I., and SØNDERGAARD, E. Acta Physiol. Scand. **22**, 299 (1951).

THE INHIBITION OF BLOOD CLOTTING BY PHOSPHATE ESTERS OF VITAMINS K AND E[1]

J. LOWENTHAL AND L. M. FISHER

Abstract

Vitamin K_1 phosphate was incubated with thromboplastin and the mixture used for the determination of the one-stage prothrombin time of human plasma. No change in thromboplastic activity was observed. The thrombin times of an artificial clotting system composed of a 1% fibrinogen solution were prolonged following incubation with vitamin K_1 phosphate. The inhibitory activity of vitamin K_1 phosphate, alpha-tocopherol phosphate, and menadiol sodium diphosphate on the one-stage prothrombin and the clotting times of rat and human plasmas was followed. Vitamin K_1 phosphate and alpha-tocopherol phosphate showed greatest activity while Synkavite showed only slight activity. At low concentrations of the phosphate derivatives the inhibition was found to be a linear function of the log of the prothrombin and clotting time. It is suggested that the phosphorylated derivatives inhibit the clotting process by interfering with the polymerization of fibrin.

Introduction

During an investigation of the antidotal activity of vitamin K_1 phosphate (2-methyl-3-phytyl-1,4-naphthohydroquinone diphosphoric ester tetrasodium salt) in vivo, an occasional transient increase in the prothrombin time immediately after the injection of the compound was observed. The plasma samples not only showed an increase in the prothrombin time but the fibrinogen formed, upon clotting, a flocculent precipitate rather than the usual fibrin gel. It has been shown previously that phosphate derivatives of various compounds interfere with enzyme systems related to the coagulation mechanism. Preston *et al.* (7) found that phosphorylated hesperidin inhibited the coagulation of blood. Bourgain and co-workers (2) suggested that this anticoagulant activity of phosphorylated hesperidin was similar to that of heparin and that the action was probably on the last stage of the clotting process, i.e., the conversion of fibrinogen to fibrin by thrombin. This observation was in agreement with the earlier reports by Ferry and Shulman (3), who found that a number of organic chemicals inhibited the action of thrombin on fibrinogen. Zierter *et al.* (9) found that one compound which showed such an effect was alpha-tocopherol phosphate. Kay and co-workers (4) later showed that alpha-tocopherol phosphate not only affected the activity of thrombin on fibrinogen but also that it inhibited the activity of thromboplastin.

Since phosphorylated vitamin K-active compounds are used as therapeutic agents in reversing the effects of indirect anticoagulants on the prothrombin time, a study was made to ascertain whether or not these preparations would

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have any inhibitory effect in vitro on the activity of thrombin and of thromboplastin. The effects of vitamin K_1 phosphate and menadiol sodium diphosphate (Synkavite, 2-methyl-1,4-naphthohydroquinone diphosphoric ester tetrasodium salt) were compared with those of alpha-tocopherol phosphate on clotting and prothrombin times of normal human and rat plasma.

Methods

Blood Samples

(a) Human.—Nine milliliters of blood were drawn from the antecubital vein, using an 18-gauge needle, into a 10-ml siliconed syringe containing 1.0 ml of 3.8% sodium citrate solution. The blood and anticoagulant were mixed and the plasma for assay was obtained by centrifuging in an angle centrifuge at 2500 r.p.m. for 15 minutes.

(b) Rat.—Blood was drawn by cardiac puncture into a siliconed syringe and immediately mixed with the citrate anticoagulant in a ratio of 9 volumes of blood to 1 volume of anticoagulant. Blood from a number of rats was pooled and the plasma was obtained as indicated in (*a*).

Clotting Times

(a) Recalcification times.—Reagents and plasma were kept in a constant temperature water bath at 37° C. To 0.2 ml of plasma was added 0.1 ml of the solution in physiological saline of the various phosphate derivatives to be tested in concentrations ranging from 0 to 1000 μg per ml. The mixture was incubated for 30 seconds before 0.2 ml of calcium chloride ($M/40$) was added. Upon the addition of the calcium chloride a stop watch was started and the tube gently tilted at 15-second intervals. The appearance of a clot was taken as the end point.

(b) Modified one-stage prothrombin time.—Reagents and plasma samples were kept at 37° C as above. To 0.1 ml of plasma was added 0.1 ml of the phosphate derivative to be tested. After 30 seconds 0.1 ml of a rabbit brain suspension was added and the mixture was incubated for a further 20 seconds before the addition of 0.1 ml of $M/40$ calcium chloride. A stop watch was started and the time to formation of a clot was recorded.

(c) Thrombin clotting time.—To 0.4 ml of 1% fibrinogen in physiological saline (0.85%) was added 0.1 ml of the phosphate derivative tested. After incubation for 30 seconds, 0.1 ml of a thrombin solution (1000 units/ml) was added, a stop watch was started and the time to the formation of a clot was measured.

Materials

Vitamin K_1 phosphate (2-methyl-3-phytyl-1,4-naphthohydroquinone diphosphoric ester tetrasodium salt) was obtained through the courtesy of Dr. Charles W. Mushett, Merck Institute for Therapeutic Research, Merck, Sharp & Dohme Research Laboratories, Rahway, N. J.

Menadiol sodium diphosphate (Synkavite, 2-methyl-1,4-naphthohydroquinone diphosphoric ester tetrasodium salt) was obtained from the Hoffmann-La Roche Company, Montreal.

Vitamin E phosphate, (*dl*-α-tocopherol monosodium phosphate) was obtained from the Warren-Teed Products Co., Columbus, Ohio.

Thrombin Topical was obtained from Parke-Davis and Co. Ltd., Brockville, Ont.

Bovine fibrinogen was obtained through the kindness of W. H. Seegers, Detroit, 90% clottable (lot No. 530225).

Results

Effect of Vitamin K_1 Phosphate on Thromboplastin Activity

When thromboplastin was preincubated with vitamin K_1 phosphate at a concentration of 5000 μg/ml for 15 to 300 seconds and the mixture used for the determination of the one-stage prothrombin time of human plasma no significant change of the prothrombin time was observed.

Effect of Vitamin K_1 Phosphate on the Thrombin Clotting Time

In order to study the effect of the phosphate derivatives on thrombin activity without the interference of other factors, an artificial clotting system was prepared consisting of a 1% fibrinogen solution in 0.85% physiological saline. The time taken for the transformation of fibrinogen to a fibrin gel after the addition of thrombin was recorded as the clotting time. Increasing concentrations of the vitamin K_1 phosphate produced a prolongation of the clotting time (Table I). It was noted also that at higher concentrations a flaky white precipitate rather than the usual compact fibrin gel was formed.

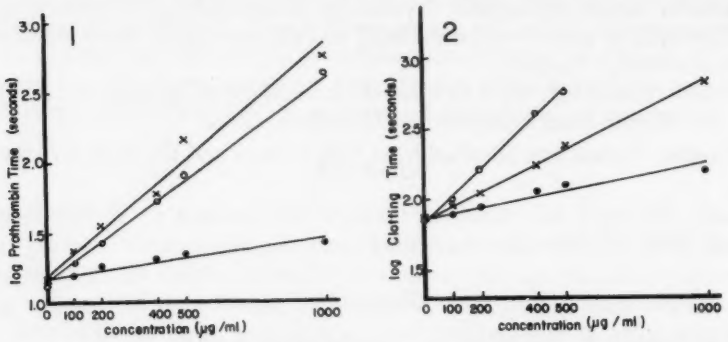
TABLE I

Effect of vitamin K_1 phosphate on action of thrombin

Thrombin (1000 units/ml)	Fibrinogen (1%)	Physiological saline (0.85%)	Vitamin K_1 phosphate (5000 μg/ml)	Clotting time (sec)
0.1 ml	0.4 ml	0.1 ml	0.0 μg	23
0.1 ml	0.4 ml	0.09	50	55
0.1 ml	0.4 ml	0.08	100	62
0.1 ml	0.4 ml	0.07	150	90
0.1 ml	0.4 ml	0.05	250	180
0.1 ml	0.4 ml	—	500	600

Effect of Phosphorylated Derivatives on the One-Stage Prothrombin Time

The effects of alpha-tocopherol phosphate, menadiol sodium diphosphate, and vitamin K_1 phosphate on the one-stage prothrombin times of rat and human plasmas were studied. Figure 1 shows the effect of the above derivatives on the prothrombin times of rat plasma. Similar results were obtained using human plasma. Vitamin K_1 phosphate and alpha-tocopherol phosphate showed greater inhibition of the prothrombin time than did menadiol



FIGS. 1 and 2. The effect of phosphorylated derivatives on the one-stage prothrombin time of rat plasma (Fig. 1) and on the clotting time of human plasma (Fig. 2).

× Vitamin K_1 phosphate.
○ Alpha-tocopherol phosphate.
● Menadiol sodium diphosphate.

sodium diphosphate. When the log of the prothrombin time was plotted against the concentration, a linear relationship was obtained for the lower concentrations of the inhibitors. At higher concentrations, however, the prothrombin times exceeded 15 minutes and a flaky white precipitate formed instead of the normal fibrin clot.

The Effect of Phosphorylated Derivatives on the Recalcification Time

The effect of alpha-tocopherol phosphate, vitamin K_1 phosphate, and menadiol sodium diphosphate on the recalcification time of human plasma is shown in Fig. 2. Similar results were obtained using rat plasma. In this assay no thromboplastin was added. At lower concentrations, the log of the clotting time was a linear function of the concentration of the phosphate derivatives. The inhibition produced by the alpha-tocopherol phosphate was greatest, that of vitamin K_1 phosphate intermediate, while that of menadiol sodium diphosphate was only slight. Higher concentrations of alpha-tocopherol phosphate and vitamin K_1 phosphate prolonged the clotting times to over 20 minutes.

Discussion

The interference with blood coagulation by vitamin K_1 phosphate was first observed in rabbits treated with indirect anticoagulants. The administration of 10 mg/kg of vitamin K_1 phosphate caused a further transient increase of the prothrombin time. This effect was followed by a gradual decrease of the prothrombin time. The biphasic effect may be explained on the assumption that the initial anticoagulant effect is due to the vitamin K_1 phosphate itself while the decrease of prothrombin time occurring in the second phase is produced by vitamin K liberated by hydrolysis of the phosphate derivatives.

It has been found that many enzyme systems require ionized calcium for optimum activity and that phosphates inhibit such systems by the removal of the calcium ion. For example, Ames (1) showed that the inhibition of the succinic oxidase system by alpha-tocopherol phosphate could be reversed by the addition of calcium chloride. The calcium ion is also essential for the normal coagulation of blood. In the present investigation, however, the addition of calcium in the determination of the recalcification time failed to reverse the effect of the phosphorylated derivatives on the clotting system.

Kay (4) has shown that the administration of alpha-tocopherol phosphate protected rats against the lethal effects of an intravenous injection of thromboplastin. Overman (6) found that prolonged incubation with high concentrations of inositol phosphatide increased the plasma prothrombin and whole blood clotting times. Bourgain (2), on the other hand, failed to detect any antithromboplastic effect of phosphorylated hesperidin using the prothrombin consumption test and therefore concluded that the anticoagulant effect was due to an inhibition of the fibrinogen-to-fibrin transformation. It was later shown by the same workers that the presence of phosphorylated hesperidin led to the formation of a structurally different fibrinogen (8). It has been shown that this transformation takes place in two stages (5). In the first, thrombin removes enzymatically a polypeptide from fibrinogen leaving a soluble fibrin monomer which in the second polymerizes nonenzymatically to an insoluble fibrin polymer. Thrombin thus acts only in the first stage of fibrin formation. We have found that the hydrolytic activity of thrombin on a synthetic substrate, TAME (*p*-toluene-sulphonyl arginine methyl ester), which may be considered to be related to the first stage, is not affected by alpha-tocopherol phosphate. It is therefore probable that the polyvalent ionic phosphate derivatives interfere with the polymer formation in the second stage and is believed to depend on steric and ionic factors.

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References

1. Ames, S. R. J. Biol. Chem. **169**, 503 (1947).
2. Bourgain, R., Symons, C., Todd, M., and Wright, I. S. J. Pharmacol. Exptl. Therap. **112**, 393 (1954).
3. Ferry, J. D. and Shulman, S. J. Am. Chem. Soc. **71**, 3198 (1949).
4. Kay, J. H. and Balla, G. A. Proc. Soc. Exptl. Biol. Med. **73**, 465 (1950).
5. Lorand, L. Physiol. Revs. **34**, 742 (1954).
6. Overman, R. S. and Wright, I. S. J. Biol. Chem. **174**, 759 (1948).
7. Preston, R. K., Avakian, S., Beiler, J. M., Moss, J. N., and Martin, G. J. Exptl. Med. Surg. **11**, 1 (1953).
8. Sheppard, E., Bourgain, R., Symons, C., and Wright, I. S. Arch. Biochem. Biophys. **50**, 224 (1954).
9. Zierter, K. L., Grob, D., and Lilienthal, J. L., Jr. Am. J. Physiol. **153**, 127 (1948).

NOTES

POLYSACCHARIDES FROM THE SAP OF THE SUGAR MAPLE (ACER SACCHARUM)[1]

G. A. Adams, C. T. Bishop, and I. J. McDonald

Although the sucrose content of maple sap has been studied extensively, no examination for polysaccharide constituents has been made. The present work was prompted by the possibility that sap polysaccharides, if they existed, might have significance as precursors of the cell wall hemicelluloses. Some properties of an arabogalactan isolated from maple sap are reported here.

Twelve samples of sap were collected from a mature maple tree between March 18 and April 11, 1958, by an aseptic technique (1). Samples of sap were plated in tryptone-glucose-extract-agar (Difco) and bacterial counts made after incubation for 5 days at room temperature. From each collection of sap an aliquot (500 ml, or the total volume if the collection was less than 500 ml) was dialyzed until free from sucrose, concentrated at $<35°$ C, and freeze-dried to yield the crude sap polysaccharides. Yields varied between 7.5 mg and 17.0 mg of polysaccharide per 100 ml of sap. Polysaccharide material (*ca.* 10 mg) from each collection was hydrolyzed by heating with *N* sulphuric acid for 10 hours at 100° C. The hydrolyzates were examined chromatographically in the solvents: (*a*) pyridine: ethyl acetate: water—1:2:2, (*b*) ethyl acetate: acetic acid: water—3:1:3, (*c*) *n*-butanol:pyridine:water—9:3:3, and the sugars were estimated quantitatively as described by Martin (2).

Arabinose and galactose were the predominant monosaccharide components of the crude sap polysaccharides and were present in an average molar ratio of 1.6:1.0 (Table I). Rhamnose was present in all samples and traces of xylose began to appear at sample 8. These latter two sugars were not present in sufficient quantities to permit their estimation and identification by other than chromatographic methods. The original crude polysaccharides all yielded varying amounts of glucose on hydrolysis, but the amount depended upon the duration of the dialysis. Prolonged dialysis (90–100 hours) against running water completely removed the glucose-containing moiety from all fractions. These results indicated that a short chain, dialyzable glucan was associated with the predominant arabogalactan type of polysaccharide in maple sap. The rhamnose-containing moiety was not removed by dialysis, and also remained with the arabogalactan when the latter was fractionated by ethanolic precipitation. These observations suggest that the small amount of rhamnose may be a structurally significant part of the predominating arabogalactan in maple sap. The crude polysaccharide material contained only small amounts of protein (1.0–1.5%) and chromatographic examination of acid hydrolyzates revealed only traces of ninhydrin-positive materials.

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TABLE I

Collection of maple sap under aseptic conditions and analysis of its polysaccharides

Sample No.	Date of* collection 1958	Volume (ml)	Microbial count per ml	Monosaccharide composition				Ratio of arabinose: galactose
				Arabinose	Galactose	Rhamnose	Xylose	
1	March 18	360	0	++++	+++	+	—	1.4:1
2	March 19	1065	0	++++	+++	+	—	1.5:1
3	March 20	2725	0	+++++	+++	+	—	1.7:1
4	March 25	1530	4	++++	+++	+	—	1.4:1
5	March 26	200	3	++++	+++	+	—	1.5:1
6	March 28	566	3	++++	+++	+	—	1.5:1
7	March 29	2915	2	++++	++	+	—	2.0:1
8	March 30	3100	3	+++++	+++	+	Trace	1.8:1
9	March 31	300	3	+++++	+++	+	Trace	1.6:1
10	April 1	2840	6	+++++	+++	+	Trace	1.6:1
11	April 2	2055	6	++++	++	+	+	1.9:1
12	April 11	218	18	+++++	+++	+	+	1.6:1

*Sap flowed only on dates shown.

There was almost complete absence of microbial growth in the sap; this observation provided assurance that the polysaccharide material was not of bacterial or fungal origin. Bacterial counts of more than 30,000,000 per ml can occur in maple sap collected without sterile precautions (1). Sap collected non-aseptically in the 1957 season contained arabogalactan polysaccharide in early samples but, as bacterial numbers increased in later collections, the polysaccharides were predominately glucan and mannan. The present results indicate that only the arabogalactan and the dialyzable glucan have their origin in the sap. Traces of xylose that appeared in the sap late in the season (Table I) probably represent a seasonal variation in sap polysaccharide because the bacterial content of these collections was judged too low to account for measurable amounts of polysaccharide.

To provide sufficient polysaccharides for further study, maple sap was collected from a commercial maple stand in 1958, the day after the trees were tapped. Bacterial counts on this sap ranged up to 3000 per ml. The polysaccharide material was prepared essentially as described above.

Paper chromatographic examination of the crude polysaccharides did not show oligosaccharides. An acid hydrolyzate of the polysaccharide contained mainly arabinose and galactose with traces of glucose, xylose, and rhamnose. Paper electrophoresis of the hydrolyzate in 0.05 *M* sodium borate did not reveal uronic acids (3). Arabinose and galactose (molar ratio 1.95:1.0) were separated by chromatography on a Celite column (4) yielding crystalline specimens of L-arabinose, m.p. 158–159° C, $[\alpha]_D^{26}$ +105°; and D-galactose, m.p. 165–166° C, $[\alpha]_D^{26}$ +80°.

The crude polysaccharide was fractionally precipitated from water by addition of ethanol (5) and was found to be heterogeneous. Electrophoretic examination of the crude polysaccharide in 0.05 *M* sodium borate in a Tiselius apparatus showed a single main peak ($\mu = 9.36 \times 10^{-5}$ cm^2 volt^{-1} sec^{-1}) and a minor, slower moving peak. Hypoiodite oxidation (6) of the polysaccharide indicated the presence of one reducing group for every 19 monosaccharide units. Graded hydrolysis (0.02 *N* oxalic acid at 100° C for 8 hours) released almost all of the arabinose units leaving the galactan portion of the molecule intact. When oxidized by periodate, the polysaccharide consumed 1 mole of oxidant per monomer unit (using 162 as the base molecular weight) and produced no formic acid.

These results indicated that the predominant polysaccharide in maple sap is an arabogalactan to which a small number of rhamnose units are attached and having a degree of polymerization of 19. Graded hydrolysis and the lack of formic acid produced on periodate oxidation showed that most, if not all, of the L-arabinose units existed in the polysaccharide in the furanoside form. The composition of the maple sap polysaccharide suggests that it is not related to the cell wall hemicelluloses of maple wood which are primarily acidic xylans (7).

A detailed structural study of the polysaccharide is now in progress and will be reported elsewhere.

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1. HOLGATE, K. C. N.Y. State Agr. Expt. Sta. (Geneva, N.Y.), Bull. No. **742**, (1950).
2. MARTIN, S. M. Chem. & Ind. 823 (1957).
3. MARTIN, S. M. and ADAMS, G. A. Can. J. Microbiol. **2**, 715 (1956).
4. LEMIEUX, R. U., BISHOP, C. T., and PELLETIER, G. E. Can. J. Chem. **34**, 1365 (1956).
5. WHISTLER, R. L. and KIRBY, K. W. J. Am. Chem. Soc. **78**, 1755 (1956).
6. CHANDA, S. K., HIRST, E. L., JONES, J. K. N., and PERCIVAL, E. G. V. J. Chem. Soc. 1289 (1950).
7. NEUBAUER, L. G. and PURVES, C. B. Can. J. Chem. **35**, 388 (1957).

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ON THE NATURE OF PORTER-SILBER CHROMOGENS SECRETED BY ISOLATED RAT ADRENALS[1]

MARION K. BIRMINGHAM AND ERIKA KURLENTS

The secretion of appreciable amounts of Porter-Silber-active lipids by rat adrenals in vitro has been reported by several authors (1, 2, 3). Heard *et al.* associated two of the four main ultraviolet-absorbing regions of chromatograms of extracts from rat adrenal incubation medium with cortisol and cortisone (4). In the present work the nature of the Porter-Silber chromogens secreted by the isolated rat adrenal in the absence of added steroids has been investigated.

Adrenals from male Sprague–Dawley rats, anaesthetized with Nembutal, were quartered and incubated in Ringer–bicarbonate–glucose solution for 2 to 4 hours with or without ACTH (U.S.P. or Connaught L-34, 100 milliunits /100 mg tissue). The methylene chloride extracts of the incubation media were developed in the toluene – propylene glycol system (5) and ultraviolet-absorption reduction of a tetrazolium derivative (May and Baker 1767) and Porter-Silber chromogen formation by the lipids eluted with methanol from the *F*, *E*, *X*, and *B* regions were compared. A detailed description of the methods used is given in a previous publication (3).

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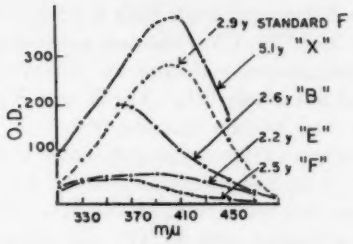

FIG. 1. Porter-Silber spectra of ultraviolet-absorbing lipids secreted by rat adrenals incubated with ACTH. The lipid extracts were developed in the toluene – propylene glycol system (5) and eluted in the regions indicated. The symbols *F*, *E*, and *B* denote the mobility of the compounds and do not imply their identification. Assigning a mobility of 1 to corticosterone (Kendall's compound B), the mobilities of lipids in the *F*, *E*, *X*, and *B* regions were approximately 0.1, 0.3, 0.6, and 1.0.

It was found that the lipid material, chiefly responsible for the formation of Porter-Silber chromogens, is located in the so-called *X* region of the toluene – propylene glycol chromatogram (4). It runs between cortisone and 11-desoxy-17-hydroxycorticosterone in this system and has not yet been identified. In Fig. 1 Porter-Silber spectra obtained with known amounts of ultraviolet-absorbing material eluted from the various regions have been compared. Eluates from the *X* region yield a typical Porter-Silber spectrum and give ratios of Porter-Silber chromogen to ultraviolet-absorbing material (PS/UV) between 0.6 and 1.0. In contrast, ratios of 0.1 are obtained with the two more polar zones, indicating that only a small proportion of these substances formed by rat adrenals in the absence of added steroids can be cortisol and cortisone despite coinciding mobilities. Sulphuric acid spectra of eluates from the *F* and *E* regions do not coincide with those of eluted cortisol and cortisone standards, while the *B* region yields a sulphuric acid spectrum identical with that of corticosterone (6).

TABLE I

Distribution of ultraviolet-absorbing material among zones of the toluene – propylene glycol chromatograms

	Zone			
	F	*E*	*X*	*B*
	μg steroid/100 mg/2 hr			
No ACTH	3.8	2.9	3.2	5.1
ACTH added	5.0	4.2	11.1	17.2
ACTH effect	1.2	1.3	7.9	12.1
No ACTH	3.4	3.7	3.3	6.0
ACTH added	4.5	4.2	12.3	27.4
ACTH effect	1.1	0.5	9.0	21.4
No ACTH	1.9	2.5	1.6	2.5
ACTH added	3.3	4.2	12.6	22.8
ACTH effect	1.4	1.7	11.0	20.3

The typical Porter-Silber spectrum with a peak at 410 mμ obtained with substance *X* and the high PS/UV ratio are surprising in view of the fact that the ratio of tetrazolium reducing to ultraviolet-absorbing material (RED/UV) was found to be only 0.1. The *E* and *B* regions yield RED/UV ratios near unity, the *F* region less than 0.1 (6).

The distribution of ultraviolet-absorbing material from rat adrenal incubates between zones *F*, *E*, *X*, and *B* is given in Table I. Fairly similar amounts are obtained from each area with medium from adrenals incubated without ACTH. When the glands are incubated with ACTH, the effect on the two more polar regions is small. The yield from the *X* region on the other hand is increased three-to nine-fold and is exceeded only by the amount eluted from the *B* region. This unidentified material represents about one third of the ultraviolet-absorbing lipid produced by the rat adrenal in vitro in response to ACTH.

Summary

The lipid-soluble, ultraviolet-absorbing Porter-Silber chromogens secreted by isolated rat adrenals in the absence of added steroids are located in the *X* region (4) of the toluene – propylene glycol chromatogram and not in the cortisone or cortisol zones. They apparently do not possess an α-ketol side chain as they do not reduce a tetrazolium derivative at room temperature. Their production is increased up to ninefold by the addition of ACTH in vitro, yielding one- to two-thirds the amount eluted from the corticosterone zone.

1. Eisenstein, A. B. Proc. Soc. Exptl. Biol. Med. **91**, 657 (1956).
2. Hofmann, F. G. Endocrinology, **60**, 382 (1957).
3. Birmingham, M. K. and Kurlents, E. Endocrinology, **62**, 47 (1958).
4. Heard, R. D. H., Jacobs, R., O'Donnell, V., Peron, F. G., Saffran, J. C., Solomon, S. S., Thompson, L. M., Willoughby, H., and Yates, C. H. Recent Progr. in Hormone Research, **9**, 383 (1954).
5. Burton, R. B., Zaffaroni, A., and Keutmann, E. H. J. Biol. Chem. **188**, 763 (1951).
6. Ward, P., Kurlents, E., and Birmingham, M. K. In preparation.

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